STUDIES

FROM THE

OTHO S. A. SPRAGUE MEMORIAL INSTITUTE

COLLECTED REPRINTS

VOLUME VII

CHICAGO
1919

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Gift of
Dr. Frank Billings

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CONTINUOUS INJECTION METHOD IN TREATMENT OF EXPERIMENTAL TUBERCULOSIS*

By

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Chicago

(Preliminary Report)

In 1915, Woodyatt of the Sprague Memorial Institute devised a machine for the continuous, prolonged and accurately timed injection of sugar. This machine has been somewhat altered and refined in the various instruments built since then, but it consists fundamentally "of a glass barrel and a metal piston driven by an electrical motor through worm and gear with a mechanically operated valve and a rheostat control. This machine was described in detail by Woodyatt in the *Journal of Biological Chemistry*, 1917, Vol. XXIX, No. 2. Woodyatt states that "if a soluble diffusible substance is made to enter the living organism at a constant rate for a long enough time, the tendency is toward the establishment of an equilibrium which is manifested by a constancy of the rate at which the organism rids itself of the substance by excretion or chemical change or both. The rate of entry is then equalled by the rate of utilization plus the rate of elimination." By the use of this instrument, Woodyatt, and Wilder and Sansum were able to determine the sugar tolerance of dogs and of human beings, both normal and diseased. They found that normal resting men and women could not utilize but began to excrete abnormal quantities of glucose, when the injection rate was over 0.8 g. and below 0.9 g. per kilogram of body weight, while the tolerance was diminished in pancreas disease, thyroid diseases and diseases of the hypophysis.

Since diuresis was caused when glycosuria was produced, the method was found useful in dehydration of certain tissues in which pressure was too high. Several cases of acute glaucoma, in which the intraocular pressure was so high that an iridectomy was unsafe, had this pressure reduced to a safe level for operation by means of a suitable selection of the rate of glucose injection and hence of the amount of water abstracted in a given time.

Other experimental work has been done on the effect of continuous injections of glucose solution at given rates on blood pressure, pulse volume, etc. Its best and so far most efficient and almost specific use has been in the feeding of cases of starvation acidosis which had reached the

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stage of acitonuria. Sansum reports a number of cases of this kind in which death was imminent because of starvation and inability to retain food administered either by mouth or rectum, but which were saved by a single or several continuous injections of glucose solutions. One of these cases was a carcinoma of the rectum with severe vomiting so that no food could be retained, several others were severe cases of colitis. In all these the effects of the continuous injection of glucose were most satisfactory. Some cases of infection with similar acidosis failed, however, to yield to the glucose treatment.

Since this method of injection of accurately timed doses of soluble substances had been proven safe for human patients, it seemed feasible to use it in chemotherapeutic experiments, especially when the therapeutic value of the drug depends on its bactericidal properties. Dr. Lewis initiated these chemotherapeutic experiments in an attempt to cure experimental pneumococcus infections in rabbits by continuous intravenous injections with ethylhydrocuprein. His report of this work was published in *Archives of Internal Medicine*, 1918, Vol. XXII, p. 593. As he states in an introductory sentence, "The effectiveness of the *in vivo* action of a chemotherapeutic agent, which is used because of its bactericidal properties, in all probability depends on the same factors which operate in the *in vitro* action of a bactericide, the two chief of which are the time factor and the concentration factor." Although Dr. Lewis was able to produce a bactericidal concentration of the ethylhydrocuprein in the rabbits' serum, he was unsuccessful in his effort to cure his rabbits infected with the virulent pneumococci. In spite of the failure in treatment of the acute pneumococcus infection by this method, it occurred to us that it might prove of value in our chemotherapeutic experiments on the much more chronic experimental tuberculosis. While bactericidal or even inhibitory power is by no means the only or even perhaps the most important factor in chemotherapy of tuberculosis, it is nevertheless one of the factors to be considered and many of the substances used have a high inhibitory value in the test tube. If it fails to inhibit the growth of the same bacilli in the animal body, may it not be because it is rapidly excreted or rapidly changed chemically, so that by the ordinary methods of administration it is impossible to maintain an inhibitory or bactericidal concentration in the blood or in the body long enough to accomplish its purpose. As noted by Lewis: "In our usual methods of administering a drug the curve representing the concentration of the substance in the blood at different intervals will be in the form of a wave. The steepness of the ascent and descent of the wave will depend on the ratio of the rate of absorption and the size of the dose to the chemical destruction + the chemical combination + the excretion. The length of time during which the drug is effective will depend on the shape of the curve and the concentration at which the drug can be effective. (See curve figured in report by Lewis cited above.) Let this figure represent the curve of concentrations which follow the intravenous injection of the largest possible dose of a drug which will not cause toxic symptoms. At once the concentration in the blood rises from zero to the point *a*. Let it

be supposed that the three factors mentioned operate so that the concentration decreases at the rate represented by the descending portion of the curve. If the concentration at which the drug is effective is at *a*, it is effective only a short time, but if it is active at *b*, it is effective much longer, and so on, the time of effective action depending on the height of the wave, the rate of its decline and the concentration of activity. An ideal drug can be pictured in this way showing that such a drug could obtain a high concentration because of its non-toxicity, would be slow in decreasing this concentration and would be highly active at a low concentration. According to the curve, if the drug was active at the concentration *b*, there are two ways in which the time of action could be lengthened: One, by repeating the original dose or fractions thereof, whenever the concentration fell to *b*. Schematically the concentration in the blood would then be represented by a series of small waves. The second way to maintain an effective concentration would be to introduce the drug continuously at a rate which would strike an equilibrium with the rate of operation of the factors which decrease the concentration. In this way, the concentration would be schematically represented by a straight line. Since Lewis of Philadelphia and DeWitt had both shown that methylene blue has a high power of inhibition of the tubercle bacillus, and since some of my chemotherapeutic experiments had indicated that the dye has some therapeutic power, methylene blue in the form used in the United States pharmacopeia was chosen for our first experiments. (For representation of machine used by us, see plates in Woodyatt's report referred to in beginning of this paper.)

In the earlier experiments rabbits inoculated with bovine tuberculo is were used, but were found unsuitable; in the later experiments, therefore, dogs having experimental bovine tuberculosis have been used. Varying concentrations of methylene blue, from 1 to 500 down to 1 to 3000, have been used, the solutions being made up with physiologic salt solution. The local toxic action in the rabbits was so severe that the ears became swollen and gangrenous and often dropped off, the animals dying often apparently from the effects of the local necrosis. The dogs injected for six hours 0.2 cc. per kilogram of body weight per minute with a concentration of 1 to 500 rarely lived more than 24 hours. The blood of these dogs, drawn immediately after the injection and added to equal parts of glycerol agar, inhibited the growth of the same strain of bovine tuberculosis as was used in the experiments, although normal blood in the same concentration gave a luxuriant growth. The inhibitory power of methylene blue alone on this strain of bovine tubercle bacillus was much less than that shown toward human tubercle bacilli, since a concentration of one part of methylene blue to 1000 parts of glycerol agar was necessary to cause complete inhibition of growth. If normal dogs' blood was added to the glycerol agar in equal parts, however, the growth was completely inhibited by 1–5000 concentration of methylene blue and almost completely by 1–50000 and 1–100000. However, since this strain seems peculiarly resistant to methylene blue, further experiments will be run, using a less resistant strain. In future experiments also, other conditions

will be modified in the effort to prolong the life of the animals, since, even with the weakest concentrations used, few of the animals have lived so long as the controls or even long enough to develop a definite tuberculosis. The experiments so far carried on, however, have given us a basis for further work and we have planned a campaign in which this method will be used for the introduction of various drugs into tuberculous animals.

The method of continuous injection seems worthy of serious consideration and it is for the purpose of introducing the method rather than of giving any results that this paper has been presented to you.

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CHEMICAL CHANGES IN TUBERCULOUS TISSUES

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In this study of the chemical composition of tuberculous tissues, the lack of uniformity in the methods used by different investigators made it imperative, for the sake of comparative results, to make analyses of normal as well as tuberculous tissues. Since large specimens were required, bovine tissues were used chiefly as large amounts of tuberculous material were available in an extensive packing center. Lymph gland and liver tubercles were used exclusively for the tuberculous specimens and normal lymph glands and livers furnished the materials for comparison. Such a comparative study reveals some of the more marked changes in the tissues, resulting from the reaction to the tubercle bacillus.

Numerous more or less complete analyses of the livers of men and of animals have been reported. The water and the fats contained in the liver received the greater share of attention from many of the earlier workers. v. Bibra[1] as early as 1849 gave 76.19% as the average water content of 6 approximately normal human livers. The amount of fat in these livers is given as 2.86% of the moist weight, or about 12% of the dry weight. Similar determinations on bovine livers gave the water content of 2 specimens as 70.86 and 71.92%, with fat percentages of 2.64 and 3.28, or calculated on the dry weight, the average percentage of fat is 10.35. In a more recent study of normal bovine liver, Profitlich[2] reported the average percentage of water in 7 different livers as 71.66. The fat content of these livers seems to have varied widely, ranging from 10.87-21.78% of the dry weight, with an average of 16.75%. The ash determined for one of these livers constituted 3.83% of the dry weight.

In connection with his study of the human liver in acute yellow atrophy and in chloroform poisoning, Wells[3] made careful analyses also of the normal human liver. He gives 77.6% as the amount of water in the normal human liver and 5% of fat. Lecithin and cholesterol determinations were made on the lipin fractions obtained from the livers of 2 persons who died suddenly. The lecithin averaged 31.7% of the ether-soluble substance, or 6.3% of the dry weight; cholesterol formed 6.7% of the ether-soluble material, or about 1.3% of the dry weight. The total amount of lecithin in the liver in acute yellow atrophy was very greatly reduced, not only as to the actual amount present, but also in relation to the other constituents of the liver. It formed

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[1] Quoted by Von Gorup-Besanez, Lehrbuch d. physiol. Chem., 1878, p. 711.
[2] Arch. f. d. ges. Physiol., 1907, 119, p. 465.
[3] Jour. Exper. Med., 1907, 9, p. 627; Jour. Biol. Chem., 1908-9, 5, p. 129.

17.0% of the ether-soluble substances, or 2.9% of the dry weight. A decrease in lecithin was also noted in delayed chloroform poisoning, although fatty changes were moderate in degree, but the decrease was by no means so marked as in acute yellow atrophy. The amount of cholesterol in the liver in acute yellow atrophy was not so greatly reduced, in fact, the total amount for the entire liver was about the same as that found in the controls, although it constituted a larger percentage of the ether-soluble substances. This is accounted for by the reduction in the amounts of neutral fats and lecithin.

In chloroform necrosis, the increase in the fat content of the liver was found to be due entirely to simple fats. As a continuation of the analyses of human liver, Wells studied also the alcohol-ether-insoluble fraction. The residue left after extraction with alcohol and ether was extracted in a shaking machine repeatedly with fresh quantities of water, and then with water at 50-60 C. The residue of liver tissue was then extracted with boiling water. The amount of gelatin in the hot water extract was determined and the remaining solids, obtained by evaporating the filtrate, were added to the united cold and warm water extracts. After the concentration of these extracts, the proteose-peptone mixture was precipitated by means of alcohol. Amino-acids and purins were determined in the nonprotein portions of the watery extracts. The residue of insoluble liver substance left after all the extractions had been completed, was dried to constant weight and analyzed. The composition of the coagulated and insoluble proteins of the liver after thorough extraction with alcohol, ether, cold and hot water, was found to be quite the same in chloroform necrosis and in acute yellow atrophy as in normal livers.

Determinations of insoluble sulphur, phosphorus and iron were made on the extracted residues. The sulphur was practically constant in all 4 specimens, in spite of the great structural changes in the 2 diseased livers. The insoluble phosphorus, however, in the acute atrophy and chloroform necrosis livers was, in each instance, increased to about 4 times the amount present in the normal livers.

The increase in the phosphorus in acute yellow atrophy is explained as the result of the great proliferative activity exhibited by the cells of the stroma and bile ducts in areas where regeneration is taking place, giving rise to large numbers of new cells rich in nucleic acid. The phosphorus found in the normal livers constituted 0.24% of the extracted residue, in acute yellow atrophy and in chloroform necrosis the phosphorus was 0.90% of this residue. The amount of sulphur ranged from 0.75-0.82% of the extracted material in the normal and pathologic livers. Heffter[4] studying the liver in relation to phosphorus poisoning, made lecithin determinations on the livers of normal rabbits and found that it constituted about 2.2% of the dry weight. In another series of rabbits which had been poisoned with phosphorus, the average lecithin content dropped to 1.12%, or about ½ that of normal livers. Baskoff[5] made lecithin and jecorin determinations on normal dog livers and on the livers of dogs poisoned with alcohol. In the normal animals, the phosphatids constituted 8.4% of the dry weight, while in animals which had been poisoned with alcohol for [illegible] months, the phosphatids had decreased to 3.9%. The jecorin in the normal livers was 14.4% of the total phosphatids, while in the animals poisoned with alcohol it had increased to [illegible]%.

[4] [illegible]

[5] [illegible]

[illegible]

Normal lymph glands, either of human beings or of the lower animals, have evidently never been made the subject of as careful studies as those recounted for the liver. In the inguinal glands of an old woman, Oidtmann[6] reported the finding of 71.43% of water, 28.45% of organic material and 0.12% of inorganic substances. In the mesenteric lymph glands of oxen, Bang[7] reported 80.41% of water, 19.59% of solids, 13.79% of total proteins, 0.69% of histone nucleinate, 1.06% of nucleoprotein, 4.76% of substances soluble in alcohol and 1.05% of mineral constituents. The structurally related thymus gland has been subjected to accurate chemical studies but here the chief interest has usually been centered in the nuclein substances. Lilienfeld[8] analyzed the cells of the calf thymus and reported a total phosphorus content of 3.01% of the dry weight; the total nitrogen was 15.03%, lecithin 7.51%, cholesterol 4.40%, fat 4.02%, and the silver salts of the nuclein bases 15.17% of the dry weight. The dry weight of these cells amounted to 11.49% of the fresh weight. An analysis of the human spleen by Burow[9] and Magnus-Levy[10] showed the water content of that organ to be 78.4%, the solids 21.53%, the fat 2.77% and nitrogen 2.79%, all calculations being made on the fresh weight of the organ. A complete analysis of the dog spleen was made by Corper.[11] The averages of the analyses of 3 normal spleens gave a moisture content of about 75-77% and a content of ether-soluble materials between 11.6 and 15.5% of the dry weight. The ether-soluble fraction was made up of about 1.5% of cholesterol, and between 6 and 7% of lecithin, leaving 2-6.5% for neutral fats. The total soluble nitrogen ranged between 0.45 and 0.97% of the dry weight and was about equally divided between that precipitable with tannic acid and that which could not be so precipitated. The water-soluble phosphorus content was about 0.27-0.52%. With the amounts of tissue used, no purins could be identified in the water-soluble fraction. The insoluble part of the tissue contained about 0.26-0.98% of dry weight as iron, 0.53-0.60% as sulphur and about 0.39% as phosphorus, with a purin nitrogen content of 0.24-0.35% of the dry weight. The total nitrogen content of the insoluble part was about 11-13% of the dry weight. Of the purin enzymes, evidence was obtained of the presence of xanthin oxidase, adenase and guanase, while uricase was lacking.

The nature of the changes occurring in tissues during the caseation produced by infections with the tubercle bacillus has been the subject of much speculation and has led to numerous investigations limited chiefly to the fatty constituents. On the basis of the gross appearance and, likewise, from the ordinary microscopic preparations, it seemed evident that caseous material was rich in fats; that it consisted chiefly of globules of fat and granules of coagulated protein. It was thought, perhaps, that the tubercle bacillus which seems to be able to synthesize fat when grown on glycerin agar, might produce somewhat similar changes in the necrotic area and its vicinity, giving rise in this way to the fatty changes of caseation.

Schmaus and Albrecht,[12] having studied caseation necrosis microscopically, state that the process consists of the death of the cellular elements and the origin of a firm intercellular substance arising as a transudate, together with the precipitation of a fibrinoid material, probably not identical with fibrin but giving the typical reaction of fibrin. The formation of the caseous detritus

[7] Hofmeister's Beiträge, 1903, 4, p. 115.
[8] Ztschr. f. physiol. Chem., 1894, 18, p. 473.
[9] Biochem. Ztschr., 1910, 25, p. 165.
[10] Biochem. Ztschr., 1910, 24, p. 363.
[11] Jour. Biol. Chem., 1912, 11, p. 27.
[12] Virchows Arch., 1896, 144, p. 72, Suppl.

follows by a progressive breaking up of the fibrinoid material. To these substances are attributed the chief importance in the formation of the firm dry condition of the caseous mass. Along with the production of the intercellular substance goes the disappearance of the chromatin of the cell nuclei. Using fat stains, Rosenthal[13] found no evidence of fat in miliary tubercles unless there was some caseation present. The fat seems to make its appearance with the occurrence of the caseation. Within an extensive caseous area no fat was found, or at most only traces, while just at the boundary of the caseous area, fat-bearing cells were seen. Some of the giant cells in this region are said to resemble fat cells, while other giant cells were apparently fat-free. Streaks containing small fat droplets were sometimes seen in sections cut through fresh caseous areas. These are explained as representing the boundaries of small caseous areas which have fused to form the larger area, in which case the fat had not disappeared as it seems to do with the gradual extension of the caseation in the tuberculous tissue. Likewise, Vallillo,[14] in studying avian tuberculosis, observed that non-necrotic tubercles composed of epithelioid and giant cells contain either no stainable fat or only sparsely grouped fat droplets in the center. In the tubercles which had necrotic centers, the fat droplets were abundant and were located chiefly in the cytoplasm of the giant cells accumulated there. The fat droplets, however, were not numerous in the caseous part of the tubercles.

Similar observations were made by Hagemeister[15] He noted that in large conglomerate tubercles, fat droplets were not infrequently observed within the tuberculous area close to the margin of the older individual tubercles. In other cases, the boundary zone apparently contained a large amount of fat. Sometimes, in the caseous tubercles, two or even three of these fat-containing marginal zones were noted at regular intervals, an occurrence explained as being due to the advance of the necrosis in successive stages. Even in the necrotic areas the outlines of the giant cells could sometimes be made out by the arrangement of the fat droplets. Herxheimer,[16] studying lung and lymph gland tubercles for the occurrence of fat, confirmed the findings of Rosenthal and Hagemeister. Chaussé,[17] using sudan III and osmic acid, demonstrated fat droplets in the giant cells and in practically all other cells in the tuberculous area and also in the substance derived from the destruction of all these cells. In a study of necrobiotic fatty changes, Joest[18] used fat stains on tuberculous tissues with results quite similar to those previously reported by Rosenthal. He was never able to demonstrate fat in the intercellular substances. Emphasis is placed on the observation that although fat is demonstrable in the caseous part of the tubercle, it is less prominent there than in the boundary zone of the living tissue, and the living tubercle tissue apart from the boundary zone is free from stainable fat. It is explained, however, that this impression of a lesser fat content in the caseous portion is, doubtlessly, due in part to the fact that the fat in the living cells of the boundary zone occurs in sharply circumscribed globules, while, in the caseous area, the globules are broken up and the fat more diffusely scattered.

[13] Verhandl. d. deutsch. path. Gesellsch., 1899, 2, p. 440.
[14] La Tubercolosi, IV, 357, 1911 [illegible].
[15] Virchows Arch., 1903, 172, p. 72.
[16] Ergeb. d. allg. Path. u. path. Anat., 1902, 8, p. 669.
[17] Compt. rend. Soc. de biol., 1908, 64, p. 377.
[18] Virchows Arch., 1911, 203, p. 451.
[19] Jour. Med. Research, 1905-6, 14, p. 491.

In the study of pathologic calcification, Wells[19] used both staining and chemical methods for the recognition of fats in tuberculous tissues. Specimens of human and bovine tuberculous lymph glands, stained with sudan III, revealed marked infiltration with fine and coarse fat granules all through the areas that are acellular, the largest and most abundant granules being usually at the periphery. When counterstained with hematoxylin, the calcium deposits were found to lie in such tissues as were stained for fat, but there was no particular difference to be noted in the amount or character of the fat in the vicinity of the calcium deposits and elsewhere. Not infrequently a calcium deposit was noted at the periphery of the gland or tubercle, while, in the center, there was no calcium but many fat granules, although not more than in the tissues surrounding the calcium deposits. For the chemical studies, Wells used tuberculous mediastinal lymph glands of cattle. He calls attention to the fact that bovine tuberculous lesions differ from human lesions in that calcification occurs during the progress of the disease and is extensive in the form of innumerable sandlike granules, scattered all through the tuberculous tissue even while the disease is in the most active stages. Calcification is usually an evidence of latency or healing in human tuberculous areas, and the deposits are found in much larger masses, each of which usually corresponds to an entire tubercle. Two sorts of bovine material were collected and examined separately in the course of his investigations. One consisted of the fluid puslike content of the large softened glands. This material escapes when the glands are opened and contains but few granules of calcium large enough to be felt by the finger. The other specimen was obtained by scraping the surface of unsoftened tubercles and the walls of the tubercle cavities. It consisted largely of the calcified material and the adherent tissue, mixed with more or less of the tissue elements but giving a fair conception of the substances immediately about the calcified masses. After drying this tuberculous material, it was thoroughly extracted with ether, alcohol and amyl alcohol. The inorganic salts of calcium were quite insoluble in these solvents. The residues left from these extractions were then extracted with large volumes of water and calcium, magnesium, phosphorus and carbon dioxid determinations made on the water-insoluble residues. The total lipin content of the scrapings from the walls of the calcified bovine lymph glands was found to be very appreciably higher than that of the caseous liquid content of these lymph gland tubercles. Since these values, in either case, are based on the dry weight, this difference is dependent in no direct way on the water content. Likewise, the low lipin content of this dried caseous material cannot be attributed to the presence of the heavy calcium salts, for both the calcium and the phosphorus are present in only about ⅓ of the amount found in the scrapings from the tubercle walls. The lipin content of calcified human tuberculous lymph glands is low, but in this case the calcium and phosphorus values are extremely high, showing that the dry weight here is made up in large part of calcium salts. The MgO was found to hold a constant relation to the CaO and the amount present was always small; likewise, a rather definite ratio existed between the amounts of carbon dioxid and of CaO. The water-soluble fraction of the caseous liquid content of the tubercles constituted a smaller percentage of the dry weight than it did in the scrapings from the walls of these tubercles. The water-soluble materials obtained from the calcified human tuberculous glands was still much smaller in amount. In the latter case this might be due to the presence of great amounts of relatively insoluble inorganic salts.

[20] Zur Chemie der Verfettung, Dissertation, Basel, 1902. Quoted by E. Schmoll, Deutsch. Arch. f. klin. Med., 1904, 81, p. 163.

Perhaps the most complete of the analyses which have been made of the lipin fraction in tuberculous caseous material are those which were made by Bossart.[20] The materials used were of human origin and only 1 consisted of completely caseous material, the remaining 4 specimens ranged from ⅙-⅓ caseous substances obtained from lymph glands. The reported fat content in percentage of dry weight varied from 13.77 in a specimen which was estimated as ⅙ caseous to 23.79 in a specimen ¼ caseous. The total fat content of the pure caseous material is given as 20.75% of the dry weight. The figures reported for the lecithin content are apparently of no great value since no lecithin at all was obtained in 3 out of the 5 specimens. In 3 specimens varying from ⅙-⅓ caseous, cholesterol ranged from 25.8-33.5% of the total fats, while the latter are reported as varying from 13.77-15.73% of the dry weight. In the completely caseous specimen, the cholesterol value is given as 2.77% of the alcohol extract, apparently a much smaller amount of cholesterol than that found in the partially caseous specimens. The negative findings for lecithin reported by Bossart have not been substantiated by other workers with caseous material. Schmoll[21] was able to demonstrate considerable amounts of glycerol-phosphoric acid in the alcoholic extracts of all specimens of pure caseous material which he examined. He used 3 specimens of completely caseous material from bovine lymph glands, and 1 specimen of human material which was about ¼ caseous. The caseous residues left after alcohol-ether extraction were extracted with cold water with the addition of toluene. This was found to be a very troublesome procedure as it was necessary to change the water twice daily for 5 or 6 weeks in order to make the extractions complete. No protein materials seemed to go into solution, as all the protein reactions were either completely negative or scarcely evident. Elementary analyses were made on the residue insoluble in alcohol, ether and water. The calculations made on the basis of the ash-free material gave the following averages for the 3 specimens of completely caseous material: carbon, 53.92%; hydrogen, 7.38%; nitrogen, 16.44%, and sulphur, 0.65%. The ash content varied from 9.2-23.3%. Phosphorus was determined only on the specimen of caseous material containing 23.3% ash and in this case it constituted 1.04% of the ash-free substance. The ash value for the human tuberculous specimen, ¼ caseous, was 4.63%, with a phosphorus content of only 0.25%. This exceptionally low percentage of phosphorus is stated as being surprising, since it was obtained from tuberculous but not completely caseated lymph glands. As this tissue is normally rich in phosphorus, this finding seemed to indicate that the process of coagulation necrosis, as appears evident also microscopically, is accompanied by a destruction of the cell nuclei and a washing away of the products formed. Whatever the sulphur content means, it was thought to be distinctly lower here than in most proteins. However, the value given is slightly higher than that given by Corper[11] for the sulphur content of the dog spleen. In order to find out more concerning the character of the protein in caseous material, Schmoll studied its conduct toward pepsin-HCl digestion or hydrolytic cleavage with HCl. The fluid obtained by about 2 months digestion with the pepsin-HCl mixture showed that this protein substance differs in no essential way from other proteins so far as its conduct toward digestion is concerned. From the results obtained, Schmoll thought that he could exclude with certainty the existence of any nuclein material whatever, since

[21] Deutsch. Arch. f. klin. Med., 1904, 81, p. 163.

no precipitate was obtained with ammoniacal silver solution and he remarks that the undissolved portion was certainly not nuclear material. Following hydrolysis with HCl, a partition of the nitrogen gave the following values:

Humin and ammonia nitrogen	5.01% of total N
Basic nitrogen	43.9 %
Amino-acid nitrogen	51.1 %

The low percentage of humin and ammonia nitrogen as well as the richness in basic nitrogen is noted as being remarkable. An attempt to determine guanin and adenin was unsuccessful. Schmoll studied also the autolysis of tuberculous caseous material and found that the autolytic processes go on extremely slowly in such tissues. He remarks that this may explain the fact that caseous material is so rarely absorbed. In connection with F. Müller, Schmoll analyzed the lipin fraction of a specimen of caseous material from human lymph glands. Cholesterol was found present but the amount is not stated. The phosphorus content of the ether-soluble material was 1.57%, or when calculated as lecithin, 38.31%. This corresponds to 3.83% of the dried caseous material.

A study of the lipoids and their content in phosphorus in different organs and tissues of guinea-pigs, during chronic tuberculosis, was made by Griniew.[22] He finds that in this disease the composition of the cells of nearly all the organs and tissues changes so far as the lipoid content is concerned. The change is qualitative as well as quantitative and is shown by the diminution in the amount of phosphorus in the lipoids and by the replacement of some lipoids by others. The total quantity of all the lipoids decreases as well. The quantity of cholesterol is increased in the liver, kidney, brain and heart, and decreased in muscles, lung, spleen and bone marrow. There is less lecithin than normal in all the organs, that is, it constitutes a smaller percentage of the total lipins. In nearly all of the organs, the percentage of kephalin is increased. The enzymes of the tuberculous tissues were also studied and the lipolytic power of the tuberculous organs was apparently subnormal. The lungs, liver and kidneys are reported as markedly subnormal in catalase, while the catalase content of the heart is increased.

A comparison of normal human livers with those of 5 persons dying of tuberculosis was made by Robin.[23] The water content was approximately 7.7% higher in the tuberculous livers than in the normal ones and this change seemed to be more marked in the acute than in the chronic forms of tuberculosis. There was only a slight change in the fat content and a minimal lessening of organic sulphur.

Corper[24] used intra vitam staining methods in a study of the fat in the tubercles of guinea-pigs. It had been shown by the work of Riddle[25] and others that fat dyes such as sudan III and scarlet R, entering the body dissolved in fat, remain either entirely or chiefly with this same food fat, being deposited with it if the food fat was deposited, but not leaving the food fat to enter either stored fat or the intracellular fats or lipoids of active tissues. Corper observed that the fats of tubercles never contained any demonstrable amount of the fat dyes administered, no matter whether the tubercles formed before or after the animal was saturated with the dye. He states, therefore,

[22] Arch. des sciences biol., 1912, 17, p. 177 and p. 363.

[23] Ztschr. f. d. ges. Physiol. d. Stoffwechs., 1911, 6, p. 576.

[24] Jour. Infect. Dis., 1912, 11, p. 373.

[25] J. Exper. Zool., 1910, 8, p. 163.

that it seems probable that the fats microscopically visible or chemically demonstrable in tubercles, are derived chiefly or solely from the existing fats and lipoids of the disintegrated cells and are not deposited from the fats in the blood. This view is entirely in harmony with the histologic evidence.

EXPERIMENTAL

Through the courtesy of the Western Packing Company and the Peerless Packing Company of Chicago, large amounts of bovine lymph gland and liver tubercles were obtained, and also fresh normal lymph gland and liver. Both the normal and the tuberculous lymph glands came from the peribronchial and mesenteric regions. The normal peribronchial lymph glands were of the usual size for cattle, the largest being about 6 cm. in greatest dimension. The mesenteric lymphoid tissue occurred in two forms: (1) lymph glands of the usual rounded or oval form with a definite hilus, and (2) long strips of lymphoid tissue, sometimes 20-30 cm. long, about 1 cm. wide and of a corresponding thickness.

From all of these lymph glands, the surrounding fatty tissues were removed with great care until no definite masses of fat were left anywhere on the surfaces of these glands. The fibrous capsules, however, were not removed and, undoubtedly, an appreciable amount of fat was left unremoved. This is noteworthy here, since the method of preparing the specimens from the tuberculous glands was such that this extraneous fat would not enter to contaminate the tuberculous materials, since, in no case, were the tuberculous glands used entirely, but only the walls of the tubercles and the caseous materials from these tubercles. The tubercles in the lymph glands averaged from 3-5 cm. in diameter and their caseous centers were sometimes 2-3 cm. in diameter. The surrounding normal tissues were removed as completely as possible from the tubercles, after which the tubercles were opened and their caseous contents expressed. The walls of the peribronchial lymph glands tubercles were kept separate from the walls of the mesenteric lymph gland tubercles, while the caseous material from all of these lymph gland tubercles was made into one composite specimen, since the amount was not sufficient to divide.

Likewise, the tubercles occurring in bovine livers were separated carefully from the surrounding liver tissue and the caseous material removed from the surrounding fibrous capsule. The largest of these tubercles varied from 1-5 cm. in diameter, while some of the livers were studded elsewhere with many smaller tubercles.

Only the larger tubercles which could be more readily separated from the normal tissues were used for these analyses. Two specimens of caseous material were obtained from tubercles having a diameter of 1-2 cm., and one specimen came from tubercles 2-5 cm. in diameter. Two specimens consisting of liver tubercle walls were preserved for analysis.

The normal and tuberculous tissues were all obtained fresh from the packing house, and all were treated throughout in as nearly as possible the same manner. The normal tissues and the walls of the tubercles were ground fine in a meat chopper, after which samples were removed for the determination of the water content and the dry weight. The caseous material consisted of a semifluid mass in which there was no macroscopic evidence of calcification other than the occurrence of numerous sandlike particles which tended readily to settle toward the bottom of the container leaving the superficial layers more distinctly fluid. The caseous material from the largest liver tubercles was more watery in its appearance than that from the smaller tubercles. Samples of the caseous material were, likewise, removed for the determination of the dry weight. The specimens of the normal and the tuberculous tissues, selected in such a way as to contain approximately 100 gm., were then weighed and about 5 volumes of 95% alcohol, containing a minimum of nonvolatile substances, were added. The specimens were then placed on a steam bath where they were allowed to remain for an hour at a temperature closely approximating the boiling point of the alcohol. Some of the specimens were used for immediate analysis, while the others preserved as indicated were kept for subsequent use.

General Plan of Analysis.—(*a*) Water content and dry weight of tissues. Amounts of the freshly ground tissue, varying from 0.5-1.5 gm., were weighed in appropriate weighing bottles and, after some preliminary drying at a lower temperature, were placed in an electric oven which was maintained at a temperature of 90-100 F. A practically constant weight was obtained by heating 48-72 hours. Some of the tissues in the weighing bottles were first treated with several volumes of 95% alcohol, which partially dissolved out the fats and rendered the tissue more porous so that a constant weight could be obtained with a briefer period of heating.

(*b*) Extraction of lipins.

When one of the preserved specimens was selected for analysis, it was first heated on the steam bath and the supernatant alcohol containing a part of the lipins was filtered through the extraction cup which was to be subsequently used in the Greene extraction apparatus. The specimen was extracted 3 times in this way with redistilled 95% alcohol. The residue was then placed on a watch glass and allowed to dry, first at room temperature and then in

the electric oven at a temperature of 90-100 F. The dried tissue was then ground in a mortar and transferred to the extraction cup which had been used previously as a filter. It was next extracted for 24 hours with hot absolute alcohol and then for 24 hours with redistilled ether. The residue was then removed from the extraction cup and the ether allowed to evaporate. The residue so obtained was once more ground in a mortar and the powdered tissue again extracted for 24 hours with absolute alcohol. All of these extraction fluids were united in a measuring flask of 500 or 1,000 c c capacity. In the early part of this work all of this lipin solution was evaporated to dryness at a temperature of 50-60 C. under reduced pressure and the total lipin fraction determined by weighing the residue so obtained. When this procedure was followed, after a nearly constant weight had been obtained in a vacuum desiccator, this lipin fraction was emulsified in water and transferred to a measuring flask of 500 or 1,000 c c, depending on the amount of fatty material obtained. Most of the lipin substances were readily transferred to the flask by means of the water emulsion; the remainder was dissolved in chloroform, which was added in small amounts up to a total volume of 25 c c. With the volume of the emulsion so adjusted that it nearly ¾ filled the flask, 10 c c of concentrated hydrochloric acid were added from a pipet while the contents of the flask were being rotated and thoroughly mixed. This usually caused a complete breaking up of the emulsion, the lipins settling to the bottom of the flask in the chloroform solution. The flask was then filled to the mark and allowed to stand until the lipins had settled out leaving a clear fluid. The supernatant acid fluid was then filtered through paper, care being taken to prevent the chloroform solution from leaving the flask. The lipin-chloroform mixture was then dissolved in hot 95% alcohol and made up to volume. This alcoholic solution of the lipins is the one which was subsequently used for the determination of cholesterol, lecithins and the iodin number. The acid solution obtained by decantation from the lipin chloroform mixture constitutes what is here called the water-lipin fraction. The volume of this fraction was noted and phosphorus and nitrogen determinations made on aliquot parts of it. The values so obtained were used in making corrections for the water-lipin solution retained by the lipin-chloroform mixture. All the phosphorus present in this water-lipin solution was considered to be inorganic phosphorus. With some of the specimens of normal tissue, the entire lipin fraction was not dried to constant weight, but instead an aliquot part, usually 1/10, was used for this purpose and a similar fraction was removed for determination of the iodin number. The remainder was evaporated to a syrup on the water bath and then emulsified with water as above indicated.

(*c*) The alcohol-ether-insoluble fraction consists of the dried residue left after extraction with alcohol and ether.

Small amounts of this residue were used for ash, calcium, total nitrogen and total phosphorus determinations. The remainder which constituted 0.7 or 0.8 of the entire amount was preserved for subsequent extraction with water.

(*d*) Water-soluble fraction.

The water-lipin solution was neutralized with sodium hydroxid and a definite portion of this used to extract a corresponding portion of the alcohol-ether residue. Either 0.7 or 0.8 of the total amount was used in each case. The neutralized water-lipin solution was divided into 2 equal parts and each in turn was used to extract the alcohol-ether residue. This extraction was accomplished in a shaking machine, each period of shaking lasting for 2 hours. Two subsequent extractions were, likewise, made using each time approxi-

mately 300 c c of distilled water. The suspended particles were removed from these extraction fluids by centrifugalizing. All four of these fluids were united, made slightly acid with acetic acid and evaporated to 1,000 c c. This constitutes the so-called water-soluble fraction, aliquot parts of which were used for the determination of the different forms of nitrogen and of phosphorus.

(*e*) Water-insoluble fraction.

This fraction is made up of the residue left after the completion of the alcohol, ether, and water extractions. It was used for the determination of total nitrogen, total phosphorus, phosphoprotein phosphorus, ash, calcium, and finally for the determination of the purin nitrogen.

ANALYTICAL METHODS

The determinations of cholesterol were made by Corper's method[26] while lecithin was estimated by the method of Koch and Woods,[27] the phosphorus being determined finally as $Mg_2P_2O_7$. The lecithin value is obtained by multiplying that of the phosphorus by the factor 25.75 on the assumption that the molecular weight of the lecithin is approximately 800. The iodin numbers of the lipin fractions were obtained by the use of von Hübl's iodin solution, the excess of iodin being titrated with 0.1 N $Na_2S_2O_3$. All total nitrogen determinations were made by the Kjeldahl method. For the estimation of the proteoses in the water soluble fraction, the proteoses were precipitated by saturation with $ZnSO_4$ in a solution made acid with H_2SO_4. The amount of nitrogen in this precipitate was then determined by the Kjeldahl method. The free amino-acids in the water-soluble fraction were estimated by the Van Slyke method.[28] For the peptones and peptids, a part of the solution was completely hydrolyzed and the total amino-acid content determined; from this total amino-acid nitrogen, the nitrogen of the free amino-acids and of the proteoses was subtracted. The negative value which was often obtained when these deductions were made serves to illustrate the unsatisfactory nature of this method of nitrogen partition. For all total phosphorus determinations, a Neumann combustion[29] was performed and the phosphorus ultimately determined gravimetrically as $Mg_2P_2O_7$.

The inorganic phosphorus was precipitated from the water soluble fraction by means of magnesia mixture, and the phosphorus determined gravimetrically as in other cases. An attempt was made to split off the phosphoprotein phosphorus in the water insoluble frac-

[26] Jour. Biol. Chem., 1912, 12, p. 197.
[27] Jour. Biol. Chem., 1905-6, 1, p. 203.
[28] Jour. Biol. Chem., 1913, 16, p. 121; and 1915, 23, p. 408.
[29] Ztschr. f. physiol. Chem., 1904, 43, p. 32.

tion by means of the action of 1% NaOH. From the solution so obtained, the phosphorus was determined in the same way as the inorganic phosphorus. The ash obtained from the alcohol-ether residues and from the water-insoluble fractions was analyzed for calcium by McCrudden's method.[30] The greater part of the water-insoluble fraction was completely hydrolyzed with 5% H_2SO_4, and the purin nitrogen estimated by the method of Krüger and Salomen.[31]

RESULTS OF ANALYSES

A comparison was made of the water content and dry weight of the normal tissues with the water content and dry weight of the caseous material and the walls of the tubercles arising from these tissues.

TABLE 1

BOVINE LYMPH GLANDS AND LYMPH GLAND TUBERCLES

Source of Tissue	Nature of Specimen	Water Content % of Moist Weight	Dry Weight % of Moist Weight
Peribronchial lymph glands	Normal	82.37	17.63
Mesenteric lymph glands	Normal	81.44	18.56
Mesenteric lymph glands	Normal	81.74	18.26
Peribronchial	Walls of tubercles	79.95	20.05
Peribronchial	Walls of tubercles	78.56	21.44
Peribronchial	Walls of tubercles	79.41	20.59
Mesenteric lymph glands	Walls of tubercles	79.51	20.49
Mesenteric lymph glands	Walls of tubercles	79.28	20.72
Peribronchial and mesenteric	Caseous material	75.16	24.84

From Table 1, it is seen that, in every case, the water content o the normal lymph glands is distinctly higher than that of the tuberculous tissues. The lower water content of the walls of these lymph gland tubercles is, doubtlessly, due in part to the replacement of the succulent normal tissue by a dense fibrous connective tissue, and, in part also, to the early deposition of calcium salts in the caseous material included within these tissues. Even the semifluid caseous material from the lymph gland tubercles has a distinctly lower water content than either the normal lymph glands or the walls of the tubercles. This is readily understood, however, when it is found that this caseous material has an ash content of about 25% of the dry weight. The presence of the heavy inorganic salts accounts for the high value for the dry weight and the corresponding low water content.

[30] Jour. Biol. Chem., 1911, 10, p. 187.

[31] Hoppe-Seyler-Thierfelder, Handbuch d. physiol. u. pathol. chem. Analyse, 1909, p. 188.

As compared with normal bovine lymph gland tissue, the water content of normal bovine liver is distinctly lower, the average in the lymph glands analyzed being 81.85% as contrasted with an average of 70.68% for liver tissue. This value for the liver is distinctly lower than that obtained from the dense fibrous walls of lymph gland tubercles, so it might be expected that when liver tissue is destroyed in tubercle formation and new connective tissue takes its place, that the water content of the tubercle walls would be higher than that of the normal tissue. This is found to be the case. The average water content of the tubercle walls was 76.93% as compared with 70.68% for the normal liver tissue. In the walls of the lymph gland tubercles, the percentage of water present was 79.35, a value approximating that obtained for the walls of liver tubercles much more closely than do the percentages obtained for the two normal tissues.

TABLE 2

WATER CONTENT AND DRY WEIGHT OF NORMAL BOVINE LIVER COMPARED WITH THE WATER CONTENT AND DRY WEIGHT OF THE CASEOUS MATERIAL AND WALLS OF TUBERCLES FROM BOVINE LIVERS

Source of Tissue	Nature of Specimen	Water Content % of Moist Weight	Dry Weight % of Moist Weight
Liver	Normal	70.60	29.40
Liver	Normal	70.67	29.33
Liver tubercles	Walls of tubercles	77.07	22.93
Liver tubercles	Walls of tubercles	76.80	23.20
Liver tubercles	Caseous material	73.00	27.00
Liver tubercles	Caseous material	74.73	25.27
Liver tubercles	Caseous material from large tubercles	78.45	21.55

The amount of water in the caseous material from the liver tubercles varies considerably in the different specimens, the extreme variation being from 73.00-78.45%. This latter value was obtained with the caseous material from only the largest of the liver tubercles, all of which were over 2 cm. in diameter. The caseous centers of these large tubercles were observed to be much more fluid than the smaller ones. The average water content for the 3 specimens of caseous material from the liver tubercles is 75.39% which agrees very closely with the percentage of water present in the single specimen of caseous material from lymph gland tubercles, namely, 75.16.

The average total lipin content obtained for the 3 separate determinations on normal bovine lymph gland tissue is 24.39% of the dry weight, or 4.4% of the most weight. The peribronchial lymph glands

gave a somewhat lower value for the total lipins than that obtained from the mesenteric glands. This difference may be explained, at least in part, by the fact that the surrounding fat could be more completely removed from the peribronchial glands. The surfaces of the mesenteric glands were always distinctly oily even after a careful removal of the closely clinging fat tissue.

TABLE 3

TOTAL LIPIN FRACTION FROM NORMAL BOVINE LYMPH GLANDS AND FROM LYMPH GLAND TUBERCLES

Source of Tissue	Nature of Specimen	Moist Weight of Specimen	Dry Weight of Specimen	Dry Weight in % of Moist Weight	Total Lipins		
					Weight in Grams	% of Dry Weight	% of Ash-free Residue
Peribronchial lymph glands	Normal	74.4	13.117	17.63	2,980	22.72	23.81
Mesenteric lymph glands	Normal	100.7	18.730	18.60	4.588	24.50	25.80
Mesenteric lymph glands (Long form)	Normal	100.7	18.317	18.19	4.756	25.96	27.29
Peribronchial lymph glands	Walls of tubercles	102.8	20.61	20.05	6.140	29.79	32.61
Peribronchial lymph glands	Walls of tubercles	102.4	21.08	20.59	6.268	29.73	32.92
Peribronchial lymph glands	Walls of tubercles	106.9	22.92	21.44	6.607	28.80	31.26
Mesenteric lymph glands	Walls of tubercles	101.2	20.74	20.49	6.180	29.80	32.21
Mesenteric lymph glands	Walls of tubercles	63.3	13.12	20.72	3.830	29.19	31.44
Peribronchial and mesenteric lymph glands	Caseous material	87.8	21.81	24.84	4.250	19.49	27.05

The fat content of the tubercle walls is strikingly constant in the 5 specimens here studied, the maximum variation being 1% of the dry weight. The average fat content of the walls of these lymph gland tubercles is 29.46% of the dry weight, as compared with 24.39% for normal lymph glands. This is an increase of more than 20%. This finding, if considered alone, would seem to substantiate the claim made on the basis of fat staining that it is the cells at the boundary of the necrotic portion of the tubercle which are especially rich in fat. This relationship is not at all definite in the liver tubercles. The caseous material from the lymph gland tubercles, on the other hand, is found to contain a much lower percentage of lipins than occurs in the walls of these tubercles. The total lipins in this specimen constituted 19.49% of the dry weight, while the average for the tubercle walls was 29.46%, or approximately ½ more. The lipin content of the caseous material was even distinctly less than was that of the normal lymph gland tissue, the average for the normal tissue being 24.39% as compared with 19.49% in the caseous material.

This is a decrease of 20% below the normal value when the calculations are made on the dry weight. However, the dry weight of the caseous material is made up in large part of calcium salts. The ash content of this specimen is about 28% as compared with 5% in the normal tissue, while the nitrogen content of the alcohol-ether insoluble fraction of the caseous material is only 67% of the nitrogen content of the normal tissue. If the calculations were made on the basis of the organic constituents of the caseous substance, there would be an actual increase in lipins in the caseous material when compared with normal lymph glands.

TABLE 4

TOTAL LIPIN CONTENT OF NORMAL BOVINE LIVER AND LIVER TUBERCLES

Source of Tissue	Nature of Specimen	Moist Weight of Specimen	Dry Weight of Specimen	Dry Weight in % of Moist Weight	Total Lipins: Weight in Grams	Total Lipins: % of Dry Weight	Total Lipins: % of Ash-free Residue
Liver	Normal	101.0	29.694	29.40	10.204	34.36	35.07
Liver	Normal	100.1	29.249	29.22	9.762	33.38	34.16
Liver	Normal	101.2	29.682	29.33	10.329	34.77	35.55
Liver tubercles	Walls of tubercles	167.6	38.421	22.93	5.848	15.23	16.68
Liver tubercles	Walls of tubercles	105.4	24.453	23.20	3.939	16.11	17.68
Liver tubercles	Caseous material	109.1	29.457	27.00	5.147	17.47	24.70
Liver tubercles	Caseous material	109.3	27.620	25.27	4.875	17.65	23.70
Liver tubercles	Caseous material large tubercles	101.7	21.916	21.55	3.971	18.12	22.74

The total lipin content of normal bovine liver forms a higher percentage of the dry weight than in normal lymph glands. The average of 3 determinations on specimens all of which came from the same liver was 34.17% of the dry weight. Three parallel determinations made on specimens from another bovine liver gave a total lipin content of 31.28%. For the 2 livers the mean value is 32.72% of the dry weight, or 9.6% of the moist weight. When compared with the normal tissue, the walls of the liver tubercles have a remarkably low content of fatty material. The average for the 2 determinations made is 15.67% of the dry weight, or less than half the amount of fat obtained from the normal tissue. This result forms a striking contrast with that obtained by extraction of the walls of lymph gland tubercles, in which the fat content was definitely higher than in the normal lymph gland tissue. A more marked contrast appears when

one compares the lipin content of the walls of the tubercles arising from livers and from lymph glands. The value obtained for the fatty fraction derived from the walls of the lymph gland tubercles is 29.46% of the dry weight; that of the walls of the liver tubercles is only 15.67%, or not much more than half as much. It is worthy of note that the walls of the tubercles from these two sources have, in each case, approximately the same dry weight and ash content, so that the difference in total lipins cannot be explained by the more abundant deposition of inorganic salts in the walls of the liver tubercles. Of the 3 specimens of caseous material, the one coming from the largest liver tubercles contains the highest percentage of fatty material, namely, 18.12% of the dry weight. This slight difference is overbalanced, however, by the fact that the ash content of this caseous material is much lower, indicating a lesser content of inorganic salts.

TABLE 5

RESULTS OF THE ANALYSES OF THE TOTAL LIPIN FRACTIONS OF NORMAL BOVINE LYMPH GLANDS AND LYMPH GLAND TUBERCLES

Source of Tissue	Nature of Specimen	Total Lipins, % of Dry Weight	Cholesterol		Lecithin		Iodin No. after Acid Precipitation	Total N, % of Lipins
			% of Total Lipins	% of Dry Weight	% of Total Lipins	% of Dry Weight		
Peribronchial lymph glands	Normal	22.72	7.93	1.80	36.05	8.49	32.8	0.92
Mesenteric lymph glands	Normal	24.50	5.73	1.40	34.76	8.52	33.9	0.89
Mesenteric lymph glands (long form)	Normal	25.96	5.89	1.53	26.01	6.75	29.9	1.06
Peribronchial lymph glands	Walls of tubercles	29.79	13.52	4.03	25.78	7.68	44.0	1.05
Peribronchial lymph glands	Walls of tubercles	29.73	13.08	3.88	24.98	7.43	45.1	1.12
Peribronchial lymph glands	Walls of tubercles	28.80	13.16	3.84	24.21	7.07	44.0	1.06
Mesenteric lymph glands	Walls of tubercles	29.80	12.30	3.67	26.52	7.98	42.8	0.91
Mesenteric lymph glands	Walls of tubercles	29.19	13.78	3.96	27.55	8.04	41.8	1.09
Mesenteric and peribronchial lymph glands	Caseous material	19.49	26.58	5.18	12.10	2.36	51.2	0.53

As determined by Corper's method, the cholesterol present in the fatty fraction from normal lymph glands represents 6.52% of it, or 1.58% of the dry weight of the specimen. The single specimen of normal peribronchial lymph glands gave a higher cholesterol value than did the specimens of mesenteric glands. From all the specimens of walls of lymph gland tubercles, the amount of cholesterol obtained remained remarkably constant, varying from 12.30-13.78% of the

total lipin fraction. The average for the 5 specimens is 13.17% of the fatty substances, or 3.88% of the dry weight. When compared with the total lipin fraction of normal lymph glands, this percentage is almost exactly twice as much, and at the same time it forms twice as large a percentage of the dry weight, indicating an actual rather than simply a relative increase in cholesterol content. This high cholesterol value for the tubercle walls is explained, at least in part, by the appreciable amounts of caseous material which could not be removed from the fibrous walls and was, therefore, included with them. This caseous material itself contains a much larger percentage of cholesterol than do the walls of the tubercles. In the single specimen here analyzed, the cholesterol constituted 26.58% of the total fatty fraction, or 5.18% of the dry weight. This is over 3 times the amount of cholesterol in normal lymph glands when calculated on the basis of dry weight.

In the estimation of the lecithin in normal bovine lymph glands, the value obtained from the specimen consisting of the long form of mesenteric glands fails to agree with the results obtained with the other specimens, and there is every reason to believe that it is distinctly too low. However, using the average of the 3 determinations, the percentage of lecithin in the total lipin fraction is 32.27, which is equivalent to 7.92% of the dry weight. In the walls of these lymph gland tubercles, the average lecithin value is 25.81% of the fatty fraction, or 7.64% of the dry weight. This is a slight but not significant decrease below that of the normal tissue. The caseous material from these tubercles contained a much smaller percentage of lecithin. It constituted only 12.10% of the total fatty fraction, or 2.36% of the dry weight. Comparing this with the results obtained with the normal tissues, the fatty substances from the caseous material contain only ⅜ as much lecithin as do the fats from the normal tissues, while on the basis of dry weight they form even a smaller relative fraction. The slight decrease from the normal value noted in the walls of the tubercles may depend in part on the caseous material included in the tubercle walls.

Iodin number determinations were made on portions of the alcoholic solution of the lipins after precipitation of the fats from the water emulsion by means of acid chloroform. While the values given in the table may have some value for the sake of comparison, they do not represent the true iodin numbers of the fats as they occurred in

the normal or in the tuberculous tissues. This is illustrated by the fact that the average of 3 iodin number determinations made on the fats from lymph glands, previous to the acid precipitation, was 41.1, while the same fats after precipitation and re-solution in alcohol gave an average iodin number of 32.2. Before this observation was made, however, all the iodin numbers had been determined on the specimens of tuberculous tissue subsequent to the precipitation of the fats in the acid solution. The iodin numbers obtained for the 5 samples of fats from tubercle walls are fairly constant and are uniformly distinctly higher than those obtained from the normal tissues. The average value is 43.5, as compared with 32.2 for the fats from the normal tissues when similarly treated. This difference is further accentuated when the fats of the caseous material are considered. The iodin number in this case was found to be 51.2.

TABLE 6

THE RESULTS OF THE ANALYSES OF THE TOTAL LIPIN FRACTIONS OF NORMAL BOVINE LIVER AND OF LIVER TUBERCLES

Source of Tissue	Nature of Specimen	Total Lipins, % of Dry Weight	Cholesterol		Lecithin		Iodin No. after Acid Precipitation	Total N, % of Lipins
			% of Total Lipins	% of Dry Weight	% of Total Lipins	% of Dry Weight		
Liver....................	Normal	34.36	2.81	0.97	39.91	13.71	43.4	0.74
Liver....................	Normal	33.38	4.11	1.37	43.00	14.35	38.9	1.07
Liver....................	Normal	34.77	4.31	1.50	40.69	14.15	40.2	0.90
Liver tubercles..........	Walls of tubercles	15.23	15.90	2.42	29.10	4.43	39.8	0.90
Liver tubercles..........	Walls of tubercles	16.11	12.97	2.09	28.33	4.56	40.3	1.16
Liver tubercles..........	Caseous material	17.47	27.26	4.75	16.74	2.91	42.1	1.02
Liver tubercles..........	Caseous material	17.65	26.05	4.61	15.71	2.78	46.0	0.93
Liver tubercles..........	Caseous material	18.12	26.20	4.75	15.45	2.80	42.3	0.88

Total nitrogen determinations were made on the lipin solutions for the purpose of showing how much nitrogen is carried over into this fraction other than that which can be accounted for by the amount of lecithin present. Calculated on the basis of 1 nitrogen atom in a molecule having a molecular weight of approximately 800, the highest amount of lecithin found in any fatty fraction would account for only 0.63% of nitrogen, while the amount actually determined was 0.92% of the total lipins. There is apparently an appreciable amount of nitrogen present in some undetermined form both in the lipins from normal and those from tuberculous tissues.

The cholesterol content of the total lipin fraction of normal bovine liver is here given as 3.74%, when the average of the three values is taken. For comparative purposes, this is probably too low since the first value given in the table is questionable because of failure to get satisfactory separation in the shaking out process.

An additional determination made on the fatty fraction from another normal liver gave the cholesterol content of the fats as 5.07%, or 1.60% of the dry weight. Including this value with those given above, the average becomes 4.07% of the total lipins, or 1.36% of the dry weight. Apparently cholesterol forms a somewhat smaller proportion of the liver lipins than it does of the lymph gland lipins, although on the basis of the dry weights there is no marked difference. The lipins from the walls of the liver tubercles are distinctly rich in cholesterol; it constitutes 14.4% of the fats, or 2.25% of the dry weight. A very much larger percentage of cholesterol, however, occurs in the lipins from the caseous material. For the 3 specimens, the average is 26.48% of the fatty fraction, or over ¼ of the entire amount. On the basis of the dry weight, the cholesterol is equal to 4.70%, or 3 times the amount obtained from normal liver. The variations in the lecithin are in the opposite direction as they were also in lymph gland tubercles. In the fats from the normal liver, lecithin constitutes 41.2%, or about 14% of the dry weight. The lipins from the walls of the tubercles contain 28.71% of lecithin, while those from the caseous material contain only 15.9%. Calculated for the dry weights, these values become 4.5% for the fats from the tubercle walls and 2.83% for those of the caseous material. Lecithin is, apparently, only about ⅜ as abundant in the lipins from the caseous material as in those from normal liver, and it constitutes only ⅕ as large a fraction of the dry weight.

The iodin number of the fats from normal liver is evidently somewhat higher than that of the fats from normal lymph glands, the average obtained for the liver fats being 40.8 as compared with 32.2 in the fats from lymph glands. For the 5 tuberculous specimens, the iodin number obtained is 42.1, or only a slight increase over that of the fats from normal liver.

The figures obtained for the nitrogen content of the lipins from the liver tissues do not differ in any definite way from those previously given for the lymph gland lipins. There is no apparent tendency for

any larger amount of nitrogen to occur in the lipin fractions from the tuberculous tissues than from the normal tissues, other than that which can be accounted for by the other lecithin percentage.

TABLE 7
Results of the Analyses of the Alcohol-Ether Insoluble Fractions of Normal Lymph Glands and of Lymph Gland Tubercles

Source of Tissue	Nature of Specimen	Alcohol-Ether Residue: Alcohol-Ether Residue in % of Dry Wt.	Total N in %	Total N in % of Ash-free Residue	Total P in %	Ash: % of Alcohol-Ether Residue	Ash: % of Dry Wt.	Calcium: % of Ash	Calcium: % of Alcohol-Ether Residue	Calcium: % of Dry Wt.
Peribronchial lymph glands	Normal	76.33	15.56	16,30	1.50	4.56	3.48	11.30	0.52	0.40
Mesenteric lymph glands	Normal	75.03	15.04	15.84	1.58	5.03	3.77	5.78	0.29	0.22
Mesenteric lymph glands (long form)	Normal	75.89	15.93	16.77	1.60	5.03	3.82			
Peribronchial lymph glands	Walls of tubercles	77.08	15.20	16.64	1.80	8.66	6.88	36.88	3.19	2.46
Peribronchial lymph glands	Walls of tubercles	76.18	13.84	15.32	2.38	9.68	7.38	47.35	4.58	3.49
Peribronchial lymph glands	Walls of tubercles	70.25	14.07	15.27	1.87	7.88	5.54	37.80	2.98	2.09
Mesenteric lymph glands	Walls of tubercles	74.44	14.52	15.70	1.83	7.48	5.57	35.20	2.63	1.96
Peribronchial & mesenteric lymph glands	Caseous material	80.80	10.41	14.45	9.88	27.95	22.58	56.27	15.73	12.71

The insoluble residue left after complete extraction of normal lymph glands with alcohol and ether averages in these specimens 75.75% of the dry weight of the tissue. The nitrogen content of this residue is 15.5% ; the phosphorus present makes up 1.56% of its weight and the ash constitutes 4.87%. The amount of calcium in the ash is not great enough for accurate determination by the method used, but it constitutes, perhaps, 0.2-0.5% of the alcohol-ether residue. The residues from the walls of the lymph gland tubercles form about the same percentage of the total dry weight as in the normal tissues, the average is 74.5% as compared with 75.75% for normal glands. The percentage of nitrogen in the residues from the tubercle walls is 14.4, while in the normal tissue it is 15.5. This decrease in the percentage of nitrogen is relatively slight when compared with the increase in ash and in calcium. The ash increases from 4.87% in normal tissue to 8.4% in the tubercle walls, while the calcium increases from less than 0.5% to an average of 3.34% of the alcohol-ether residue. The change in the phosphorus content by no means parallels that of the ash.

Its increase is from 1.56-1.97% of the residue. If the amount of calcium found here is combined in the usual way with phosphoric and carbonic acids in the approximate ratio of 4:1, about 1.5% of the phosphorus present would be required to unite with the calcium, leaving only about 0.5% for the organic compounds.

The specimen of caseous material is conspicuous for its high ash and calcium content, in spite of the fact that there were no definitely calcified areas in any of these tubercles. The ash constituted 27.95% of the alcohol-ether residue, or calculated on the basis of the dry weight this is equivalent to 22.58%. The calcium itself made up 15.73%, or approximately 1/6 of the alcohol-ether residue, while the phosphorus constituted 9.88% of it.

TABLE 8

THE RESULTS OF THE ANALYSES OF THE ALCOHOL-ETHER INSOLUBLE RESIDUES FROM NORMAL LIVER AND FROM LIVER TUBRCLES

Source of Tissue	Nature of Specimen	Alcohol-Ether Residue								
		Alcohol-Ether Residue in % of Dry Wt.	Total N in %	Total N in % of Ash-free Residue	Total P in %	Ash		Calcium		
						% of Alcohol-Ether Residue	% of Dry Wt.	% of Ash	% of Alcohol-Ether Residue	% of Dry Wt.
Liver..........	Normal	68.19	14.95	15.26	0.64	2.03	1.39	19.05	0.39	0.27
Liver..........	Normal	69.25	14.91	15.26	0.69	2.28	1.58	15.80	0.36	0.25
Liver..........	Normal	67.07	15.27	15.51	0.69	2.19	1.47	22.20	0.44	0.30
Liver tubercles	Walls of tubercles	86.94	14.53	15.91	1.82	8.69	7.56	39.45	3.43	2.98
Liver tubercles	Walls of tubercles	88.18	14.61	16.04	1.86	8.90	7.85	43.79	3.90	3.44
Liver tubercles	Caseous material	83.76	10.15	14.35	5.06	29.27	24.52	54.29	15.89	13.31
Liver tubercles	Caseous material	85.49	10.66	14.31	4.82	25.53	21.65	51.07	12.93	11.05
Liver tubercles	Caseous material (large tubercles	82.82	13.32	16.71	3.59	20.30	16.81	50.50	10.25	8.49

In the 3 specimens of normal liver, the residues left after extraction with alcohol and ether average 68.16% of the dry weight.

Because of the low fat content in the walls of the liver tubercles, an especially high value is obtained for the alcohol-ether insoluble fraction. This forms 87.56% of the dry weight, as compared with 68.16% in the normal tissue. The residues from the caseous material form a slightly smaller percentage of the dry weight, than do the residues from the tubercle walls.

In the normal liver tissue, nitrogen constitutes about 15% of the alcohol-ether residue. Its amount is slightly lower in the walls of the

tubercles where it averages about 14.5%. In the 2 specimens of caseous material from medium-sized liver tubercles, nitrogen forms 10.4% of the residue, although the inorganic materials forming the ash make up 27.4% of this fraction. This value for the nitrogen if calculated on the basis of the organic substances present would form 14.3% of such compounds, which shows that there is no marked reduction in the amount of nitrogen in this caseous material below that which would be present in a corresponding amount of protein under normal conditions. The reduction in the amount of nitrogen is still less in the specimen of caseous material from the large liver tubercles. Here, it constitutes 13.2% of the alcohol-ether residue, although the ash in this case formed ⅕ of the entire weight of this fraction.

TABLE 9

THE RESULTS OF THE ANALYSES OF THE ALCOHOL-ETHER-WATER INSOLUBLE RESIDUES OF NORMAL LYMPH GLANDS AND LYMPH GLAND TUBERCLES

Source of Tissue	Nature of Specimen	Water Insoluble Residue: Water Insol. % of Dry Wt.	Total N in %	Total N in % of Ash-free Residue	Total P: % of Water Insol.	Total P: % of Dry Wt.	Phosphoprotein P, % of P	Ash: % of Water Insol.	Ash: % of Dry Wt.	Calcium: % of Ash	Calcium: % of Water Insol.	Purin N % of N
Peribronchial lymph glands	Normal	67.97	16.12	16.36	0.60	0.41	0.14	1.44	0.98	13.04	0.19	0.32
Mesenteric lymph glands	Normal	69.43	15.52	15.85	0.95	0.66	0.18	2.11	1.46	11.69	0.25	0.57
Mesenteric lymph glands (long form)	Normal	68.79	16.01	16.30	1.02	0.70	0.16	1.78	1.22	6.67	0.12	0.44
Peribronchial lymph glands	Walls of tubercles	72.68	14.46	15.47	1.52	1.10	0.53	6.50	4.72	58.79	3.82	0.21
Peribronchial lymph glands	Walls of tubercles	68.07	14.44	15.80	1.37	0.93	0.65	8.61	5.86	46.08	3.97	0.26
Peribronchial lymph glands	Walls of tubercles	62.61	14.08	15.03	1.16	0.73	0.69	6.30	3.94	43.97	2.77	0.22
Mesenteric lymph glands	Walls of tubercles	63.97	15.65	16.43	1.34	0.86	0.88	4.73	3.03	28.57	1.35	0.28,
Peribronchial & mesenteric lymph glands	Caseous material	78.22	10.64	15.81	5.07	3.97	2.04	32.68	25.56	41.13	13.44	0.06

The value obtained for the total phosphorus in the alcohol-ether insoluble fraction of normal liver is 0.67%. In normal lymph glands, the corresponding percentage was 1.56%. In the walls of the liver tubercles, the amount of phosphorus increased to nearly 3 times the amount found in the normal tissue, while the calcium present increased to nearly 10 times that obtained from normal tissue. An exceptionally high phosphorus content occurs in the residues from the caseous material. In these the phosphorus averaged 4.49%, or 7 times that

of normal liver. With this increase in phosphorus, there is much more than a corresponding increase in the amount of ash. For the normal liver, the ash value is about 1.5%, while in the caseous residues, it averages approximately 21%, or 14 times the amount in the normal tissue. The increase in calcium more than parallels the increase in ash; from a normal of 0.4%, it increases to 3.6% in the tubercle walls, and reaches 13% in the caseous residues.

A comparison of the alcohol-ether residues of the specimens of normal lymph glands with the water insoluble fractions makes evident the fact that only a small percentage of the residues goes into solution in water at room temperature. In the water insoluble fraction, there is a decrease of 7.02% of the dry weight below the percentage of the alcohol-ether residue. In the specimens from the walls of the lymph gland tubercles, the decrease is 7.65%, and for the caseous material 2.58%. The water soluble materials form, apparently, a smaller percentage of the caseous substances than they do of the normal tissues or of the tubercle walls. In nearly every case, the total nitrogen present in the water insoluble residues constitutes a slightly higher percentage than it does of the alcohol-ether residues. The loss in weight is evidently due to the solution of substances relatively poorer in nitrogen than those which remain. As a result of the extraction with water, the residues suffer a loss in their phosphorus content. This is a decrease of approximately 45% in the normal tissues and about 40% in the tuberculous specimens. That portion of the phosphorus which is split off from the residues by 1% NaOH is here listed as phosphoprotein phosphorus. In the normal tissues this constitutes about ⅕ of the total phosphorus, while in the tuberculous specimens it forms a much larger part of the total phosphorus, sometimes even more than half. These high values are, doubtlessly, due to a solution of a part of the inorganic phosphorus when the alkaline solution is neutralized with acetic acid. The percentages of ash and calcium are usually somewhat lower in the water insoluble fractions than in the alcohol-ether residues. The determinations of purin nitrogen in the water insoluble residues of normal lymph glands gave an average of 0.44% of these residues, while there was a distinctly smaller amount in the tubercle walls where the percentage was 0.24%. The single specimen of caseous material from lymph glands seemed to contain only a trace of purin nitrogen, the value obtained being 0.06% of the water insoluble fraction. This low content of purin nitrogen in

caseous material is not surprising, but it stands out in striking contrast to the exceptionally high percentages obtained from the caseous material from liver tubercles.

TABLE 10

The Results of the Analyses of the Water-Insoluble Residues from Specimens of Normal Liver and from Liver Tubercles

Source of Tissue	Nature of Specimen	Water Insoluble Residue										
		Water Insol. % of Dry Wt.	Total N in %	Total N in % of Ash-free Residue	Total P		Phospho-protein P, % of P	Ash		Calcium		Purin N % of N
					% of Water Insol.	% of Dry Wt.		% of Water Insol.	% of Dry Wt.	% of Ash	% of Water Insol.	
Liver..........	Normal	65.91	15.20	15.26	0.25	0.17	0.09	0.39	0.26			0.15
Liver..........	Normal	65.48	15.12	15.17	0.27	0.18	0.06	0.34	0.22			0.14
Liver..........	Normal	64.17	15.22	15.39	0.24	0.16	0.06	1.08	0.71	55.56	0.60	0.10 0.11
Liver tubercles	Walls of tubercles	81.52	13.99	15.56	2.26	1.84	1.03	10.08	8.22	54.39	5.49	0.16
Liver tubercles	Walls of tubercles	81.18	14.17	15.43	1.51	1.23	0.83	8.19	6.65	49.49	4.05	0.16 0.14
Liver tubercles	Caseous material	76.63	10.64	15.14	5.34	4.09	2.34	29.75	22.80	59.08	17.58	0.26
Liver tubercles	Caseous material	80.14	10.97	15.07	4.57	3.66	2.39	27.21	21.81	61.71	16.79	0.27
Liver tubercles	Caseous material (large tubercles)	79.66	13.05	15.48	3.58	2.85	2.15	15.69	12.50	40.15	6.30	0.27

In the specimens of normal bovine liver, the water insoluble fractions form 65.18% of the dry weight, while the alcohol-ether residues constitute 68.17%. As a result of the extraction of these residues with water, the amount which goes into solution, together with a small mechanical loss, is 3% of the dry weight of the specimen.

From the residues of the tubercle walls, 6.21% of the dry weight passed over into the water soluble fraction, and from the residues of the caseous material, 5.21%. Here, as in the lymph gland tissues, the percentage of nitrogen in the water insoluble residues is slightly higher than in the alcohol-ether residues. In the normal specimens the total phosphorus is reduced to less than half that of the alcohol-ether residues, but there is no corresponding reduction in the phosphorus in the specimens from tuberculous tissues; in these, the phosphorus values remain practically unchanged. The amount of ash, likewise, is decreased in the normal specimens, but remains nearly constant in the tuberculous residues. Calcium, as a rule, forms a larger percentage of the water insoluble fraction than it does of the alcohol-ether residues. The average value obtained for the purin

nitrogen in the residues of normal liver is 0.13% of the water insoluble fraction; in the residues from the tubercle walls, a similar percentage is obtained, namely, 0.15. Very strangely, three closely agreeing determinations of purin nitrogen made on the residues of caseous material from liver tubercles gave a distinctly higher purin content than that of normal liver or of the liver tubercle walls. Here, the percentage obtained was 0.27, as compared with 0.13 for normal liver and 0.15 for the tubercle walls.

TABLE 11

THE RESULTS OF THE ANALYSES OF THE WATER SOLUBLE FRACTION OF NORMAL BOVINE GLANDS AND LYMPH GLAND TUBERCLES

Source of Tissue	Nature of Specimen	Water Soluble Fraction: Nitrogen: Total N in % of Dry Weight	Pro-teose N in % of Total N	Am-monia N in % of Total N	Free Amino-Acid N in % of Total N	Amino-Acid N in % of Total N	Phosphorus: Total P in % of Dry Weight	Inor-ganic P in % of Total P
Peribronchial lymph glands	Normal	1.59	7.81	7.38	21.10	27.59	0.88	54.54
Mesenteric lymph glands	Normal	1.28	9.03	1.00	23.59	36.48	0.74	56.20
Mesenteric lymph glands	Normal	1.41	9.37	Trace	14.33	30.85	0.66	50.21
Peribronchial lymph glands	Walls of tubercles	0.89	16.90	7.48	21.40	28.33	0.50	78.83
Peribronchial lymph glands	Walls of tubercles	0.88	12.90	5.16	15.80	33.12	0.53	88.26
Peribronchial lymph glands	Walls of tubercles	0.83	11.65	2.22	28.68	38.20	0.48	85.89
Mesenteric lymph glands	Walls of tubercles	1.66	24.58	7.80	18.61	36.40	0.59	80.21
Peribronchial and mesenteric lymph glands	Caseous material	0.26	35.20	Lost	14.89	26.80	0.20	93.50

The total nitrogen in the water soluble fraction from the specimens of normal lymph glands is equivalent to 1.43% of the dry weight of the specimens. The percentage of nitrogen in the corresponding fractions from the walls of the peribronchial lymph gland tubercles is, in each case, definitely lower than in the normal tissues, the average being 0.87% instead of 1.43%. The tubercle walls from the mesenteric lymph gland tubercles appear, from Table 11, to be exceptional in their high content of water soluble nitrogen, but this is explained by the fact that a clear solution was not obtained by centrifuging and some protein material was carried over in suspension.

The amount of water soluble nitrogen in the specimen of caseous material is exceptionally low, constituting only 0.26% of the dry

weight, or less than 1/5 of that derived from the normal tissue. There seems to be a rather definite increase in the proteose nitrogen in the tuberculous as compared with the normal tissues, the caseous material being the richest in this form of nitrogen compounds. The values obtained for ammonia nitrogen are quite inconstant, as are those, also, for the free amino-acids. The attempt to determine a peptone nitrogen fraction, following acid hydrolysis of samples of the water soluble substances, was eminently unsatisfactory. The value obtained for the peptone nitrogen was often a negative one. A slightly smaller percentage of phosphorus, when calculated on the basis of the dry weight, goes into the water solution from the tuberculous than from the normal tissues. This difference is most marked in the specimen which was completely caseous. On the other hand, of the phosphorus which does enter the water fraction, an increasing large percentage of it is inorganic phosphorus in the specimens of tuberculous tissues.

TABLE 12

THE RESULTS OF THE ANALYSES OF THE WATER SOLUBLE FRACTIONS OF NORMAL BOVINE LIVER AND OF LIVER TUBERCLES

Source of Tissue	Nature of Specimen	Water Soluble Fraction						
		Nitrogen					Phosphorus	
		Total N in % of Dry Weight	Proteose N in % of Total N	Ammonia N in % of Total N	Free Amino-Acid N in % of Total N	Amino-Acid N in % of Total N	Total P in % of Dry Weight	Inorganic P in % of Total P
Liver	Normal	0.75	5.44	2.43	18.35	32.20	0.49	66.25
Liver	Normal	0.81	2.11	7.11	11.50	23.84	0.56	58.91
Liver	Normal	0.96	4.27	0.42	9.21	29.49	0.59	57.61
Liver tubercles	Walls of tubercles	0.68	39.19	5.10	16.10	41.45	0.26	77.58
Liver tubercles	Walls of tubercles	0.71	37.45	5.42	18.23	34.80	Lost	Lost
Liver tubercles	Caseous material	0.24	13.37	10.72	37.46	40.05	0.17	84.51
Liver tubercles	Caseous material	0.23	26.12	9.87	38.38	43.30	0.14	Lost
Liver tubercles	Caseous material (Large tubercles)	0.29	14.01	Lost	23.57	27.87	0.21	80.88

In liver, as well as in lymph gland specimens, the higher percentage of water soluble nitrogen is obtained with normal rather than with tuberculous specimens. The difference, in this respect, between normal liver and the walls of liver tubercles is slight, but only about 1/3 as much nitrogen goes into solution from the caseous material. The ammonia nitrogen fraction is apparently somewhat increased in the

caseous material from liver tubercles, as is also the free amino-acid content. The value obtained for peptone nitrogen is negative in every specimen of tuberculous liver, although it has a positive value in the normal tissues. The phosphorus entering the water solution from the tuberculous tissues, in every case, constitutes a smaller percentage of the dry weight than with normal tissues.

Here, again, an increasingly large percentage of the total phosphorus is inorganic phosphorus in the water solutions from the tuberculous tissues.

THE ANALYSIS OF CASEOUS MATERIAL FROM HUMAN LYMPH GLAND TUBERCLES

This specimen consisted of the caseous material from 3 tracheobronchial lymph glands. The largest of these glands was about 3.5 cm. in its greatest dimension and it was completely caseous without any definite areas of calcification in it, other than fine sandlike particles. The two smaller glands were caseous and partly calcified. The entire specimen weighed only 13.5 gm. and was, therefore, too small for accurate analysis.

In spite of the fact that most of the specimen formed a semifluid mass, its dry weight was 60.7% of the moist weight, or the water present formed only 39.3% of the original weight. From the entire specimen 0.45 gm. of lipins was obtained, which is equivalent to 5.5% of the dry weight, or 19.7% of the ash-free residue. Unfortunately, the cholesterol was lost. Lecithin was found to constitute 30.9% of the total lipins, a percentage 2½ times as great as that of the fats from bovine caseous material. This lecithin value represents 1.7% of the dry weight, or 6.1% when calculated on the ash-free basis.

The iodin number of these fats was found to be 30.7, which is about the same as that obtained for the fats from normal bovine glands, but much lower than that from the single specimen of caseous material.

The alcohol-ether residue of this specimen formed 93% of the dry weight, and the water-insoluble fraction 88.9%. The total nitrogen determinations made on the alcohol-ether residue and on the water-insoluble fraction gave 2.46 and 2.33% of nitrogen, respectively. When the ash content of these residues has been deducted, these nitrogen values become 10.93 and 10.84%. This represents a reduction of the organic substances far below that seen in any of the specimens of caseous material from bovine tissues. The total phosphorus content of each of these residues was 9.25-9.50%, while the

ash constituted 77.5-78.5%. Of this ash the calcium formed over 60% of its weight. An attempt was made to evaluate the purin nitrogen, but evidence of only a trace of purins was obtained.

DISCUSSION OF RESULTS

The results of the analyses of normal bovine lymph glands agree closely, so far as they are comparable, with those obtained by Bang.[7] For the water content and dry weight of mesenteric glands of oxen, he reported 80.41% of water and 19.59% of solids, as compared with the figures here given of 81.59% of water and 18.41% of solids for glands having the same origin. There is a similar close agreement on the percentage of fatty substances present. Bang gave the alcohol soluble substances as 4.76% of the fresh weight; they are here reported as constituting 4.49%, or the equivalent of 24.39% of the dry weight. The percentage of ash given by Bang is 1.05%, a value somewhat higher than that obtained in these analyses. The water content of normal bovine liver as given by v. Bibra[1] is 71.39% of the moist weight and the average of Oidtmann's determinations is 71.66%. For the two livers examined in these analyses, the water content was found to be 70.63%, leaving a dry weight of 29.37%. No such close agreement exists with regard to the fat content of bovine livers. v. Bibra gives percentages of 2.64 and 3.28, based on the fresh weight, or when calculated on the dry weight, the average is 10.35. Profitlich found the fat to vary from 10.87-21.78% of the dry weight. In the two normal livers which I have examined, the total lipins constituted 31.28 and 34.17% of the dry weight, or an average of 32.72%. There was no macroscopic evidence of pathologic fatty changes in either of these livers and the higher percentages obtained are probably due to the method of extraction.

In the tuberculous tissues, the finding of a higher fat content in the walls of lymph gland tubercles than in the completely caseous material agrees with the observations made by Wells in regard to the scrapings from tubercle walls, but differs in the fact, that in this caseous material both calcium and phosphorus were present in much larger amounts than in the tubercle walls, so that a part of the decrease in total lipins may be attributed to the deposition of calcium salts. His finding that the water soluble fraction of the caseous liquid content of tubercles constitutes a smaller percentage of the dry weight than it does in the scrapings from the walls is confirmed by these analyses. A similar low lipin content was found for the caseous material from human lymph glands, but here there is a correspond-

ingly great increase in inorganic salts and a like decrease in the protein constituents. Bossart obtained about the same amount of fat from pure caseous material from human lymph glands as was obtained here for the caseous material of bovine origin. The values which he reports for his partially caseous specimens are lower, however, than those reported here for the walls of tubercles or for normal lymph gland tissue. According to Bossart's analyses, cholesterol apparently made up a larger percentage of the total fats in the partially caseous material than it did in the completely caseous specimen. In bovine lymph glands and livers, cholesterol seems to constitute a much larger percentage of the fats from the caseous material than it does of the fats from the walls of tubercles or from normal tissues.

The variation in the amount of lecithin is in the opposite direction. It is more abundant in the specimens of normal tissue than it is in the tuberculous ones and the amount in the caseous material constitutes the smallest percentage of the total fats, as well as of the dry weight. This finding of a decrease in the lecithin content of the fats from the caseous material harmonizes with the similar finding by Wells in his study of the fats of livers in acute yellow atrophy and in delayed chloroform poisoning, and also with the observation made by Griniew on the organs of tuberculous guinea-pigs.

So far as lymph gland tubercles are concerned, the results obtained by these analyses seem to support the evidence furnished by staining methods that the walls of tubercles contain a larger amount of fat than does the caseous material itself. While this does not hold true of liver tubercles, it seems quite likely that the difference is due to the more rapid formation of the tubercles in the liver tissue which is already extremely rich in fats. In all 4 specimens of caseous material, the total lipins constitute a smaller percentage of the dry weight than in the normal tissues from which this caseous material originated. This shows conclusively that caseous material is not so rich in fats as it has usually been considered.

The cholesterol of the total lipins increases at about the same rate that the lecithin decreases in the tuberculous tissues, so that the sum of the 2 percentages remains practically constant, leaving the simple fats to form about the same percentage of the total lipins in normal and tuberculous tissues.

In the alcohol-ether-insoluble residues, the percentage of total nitrogen is slightly higher in the normal lymph gland residues than in those from normal liver, in spite of the fact that ash content of the

lymph gland residues averages twice that of the normal liver residues. The walls of the tubercles arising from these 2 tissues give residues which agree more closely in their nitrogen content than do the tissues from which they arise, just as they also resemble each other more closely in their histologic structure. The residues from 3 of the specimens of caseous material give approximately the same percentage of nitrogen, and about the same percentage of ash, whether from lymph gland or from liver tubercles. In the residues from the caseous material of the large liver tubercles, the higher content of nitrogen is dependent in part on the smaller amount of inorganic salts present, and probably in part also on the more rapid necrosis than that which occurs in the formation of the smaller tubercles, so that less extensive changes have taken place in the proteins originally present in the area.

The total phosphorus content of the normal lymph gland residues averages twice that of the residues of the normal liver. This can be explained by the greater amount of nucleoproteins in the lymph glands. The walls of the tubercles arising in lymph glands or in bovine liver give residues which contain approximately the same amount of phosphorus. As compared with the phosphorus content of the normal tissues, the increase in the amount of phosphorus in the walls of the lymph gland tubercles is small as compared with the increase in the ash content. This is apparently due to a decrease in the nucleoproteins and their replacement by proteins poorer in phosphorus, together with the deposition of inorganic salts. In the residues from liver tubercles, the amount of phosphorus is increased to nearly 3 times the amount in normal tissue, although the total ash content is only slightly higher than that of the walls of lymph gland tubercles. In this case there was no tissue rich in nucleins to be replaced, so that the increase in the phosphorus is due chiefly to the deposition of inorganic salts.

In the water-insoluble residues of lymph glands and lymph gland tubercles, the purin nitrogen decreases with the tubercle formation and reaches a minimum in the residues of caseous material. As lymph gland tissue is replaced by fibrous tissue relatively poor in nuclein substances, a decrease in purin nitrogen would be expected in the tubercle walls. Likewise, in caseation, as the nuclear substances disappear, as shown by staining methods, a further reduction of purin content probably also occurs. From the results obtained with residues from normal liver and from liver tubercles, the tubercle walls are

apparently slightly richer in nucleoproteins than is the normal liver. A finding which, at present, cannot be explained is the distinctly greater purin content of the caseous residues of liver tubercles as compared with the purins in the residues of normal liver and in walls of liver tubercles. Three closely agreeing determinations give an average value ½ more than that of the tubercle walls and approximately twice that of normal liver tissue. This does not conform with the finding of an extremely low percentage of purin nitrogen in the single specimen of caseous material from bovine lymph gland tubercles.

SUMMARY

The water content of normal bovine lymph glands constitutes about 81 or 82% of the moist weight. No very distinct differences are noted between peribronchial glands and those from the mesenteric region. The tubercle walls and the caseous material from lymph gland tubercles contain a lower percentage of water than does the normal tissue.

In normal bovine liver tissue, the percentage of water present is less than that of the tubercle walls or of the caseous material from liver tubercles. The specimens of caseous material from lymph gland and liver tubercles approach each other closely in their water content, the average being about 75% for the bovine material.

The alcohol-ether-soluble substances from normal bovine lymph glands form about 24.4% of the dry weight, or about 4.4% of the moist weight. The walls of the lymph gland tubercles contain a distinctly larger amount of lipins than does the caseous material or the normal tissue. On the contrary, the walls of liver tubercles are poor in lipins as compared with the normal tissue, and they contain a smaller amount of fats than does the caseous material from these tubercles. When calculated on the basis of the dry weight, the caseous material from lymph gland tubercles contains a smaller percentage of lipins than does normal lymph gland tissue. When the ash is deducted, this difference disappears and the content of lipins becomes equal to or slightly greater than that of the normal tissue, but less than that of the tubercle walls. When calculated on an ash-free basis, the lipin content of the caseous material from liver tubercles is distinctly less than that of the normal tissue but greater than the lipin content of the tubercle walls.

Cholesterol forms about 6.5% of the lipins from normal bovine lymph glands, or about 1.5% of the dry weight. The lipins from the walls of lymph gland and liver tubercles contain, in every case, 2-3

times as much cholesterol as do the lipins from the normal tissues. This is an actual increase also when calculated on the basis of the dry weight. The caseous material contains even a larger percentage of cholesterol than do the tubercle walls.

Lecithin constitutes about 32% of the lipin fraction of normal bovine lymph glands, or about 7.9% of the dry weight; the corresponding values for normal liver are 41.2% of the fats, or 14% of the dry weight. The lecithin content of the fats from the tubercle walls is slightly less than that of the normal tissues, while there is a very marked reduction in the lecithin content of the lipins from caseous material of bovine origin. In the specimen of caseous material from human lymph glands, lecithin formed 30.9% of the total lipins.

The iodin numbers obtained for the fats of the tuberculous specimens from lymph glands are higher than those from the normal tissues. This observation does not hold true for the liver specimens. In the latter, there is no difference noted between the iodin numbers obtained for the lipins from normal and tuberculous specimens, although the values are practically the same as those from the fats from the lymph gland tubercles.

In the residues of caseous material left after extraction with alcohol and ether, the nitrogen content remains relatively high, in fact, the reduction in nitrogen content is only slight when the calculations are made on ash-free residues. The percentage of nitrogen does not differ much from that obtained from the normal proteins of these tissues.

In specimens of caseous material in which there are no macroscopic evidences of calcification other than the presence of sandlike particles, calcium sometimes forms as much as 15% of the residue left after extraction of the fats. In such residues, the phosphorus content may reach 9%.

The amount of purin nitrogen in the walls of lymph gland tubercles is only slightly more than half that of normal lymph gland tissue, and the amount is apparently much less in the caseous material. In the residues from the walls of liver tubercles, purin nitrogen is present in only slightly higher percentage than in the normal liver. The results here obtained would seem to indicate that the purins are even more abundant in the caseous residues of liver tubercles.

The amount of material which enters the water solution during extraction is distinctly less from caseous material than from the residues of normal tissues.

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AN INVESTIGATION OF THE ACID FASTNESS OF TUBERCLE BACILLI (I)

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Very soon after Koch's discovery of the tubercle bacillus, investigators became interested in its peculiar staining reactions, especially in the "acid fastness" of this microörganism; and this question has occupied investigators up to the present time. Why are these bacilli acid fast? How can this characteristic be modified? Is there any relation between the staining reactions and the virulence of the microorganisms? These are some of the questions which have occupied investigators.

Ehrlich, Klein and Marmorek all noted the interesting observation that young tubercle bacilli are not acid and alcohol fast, a fact which Marmorek explains by the hypothesis that they have not yet had time to develop the fatty and waxy capsule, the impermeability of which was supposed to be the cause of the characteristic staining reactions. Koch, Borrel, and others have succeeded in extracting acid fast substances from the tubercle bacillus, thus robbing it of its acid fastness without, according to Borrel, robbing it of its power to develop tubercles. Wherry, in experiments with a strain of tubercle bacilli which had become saprophytic, probably from long continued cultivation on artificial media, was able to modify the acid fastness by varying the media on which the organism was grown. He states that the culture could be rendered non-acid fast by continued growth under conditions unfavorable to the synthesis of fats, and that the culture could synthesize fatty bodies rendering it acid proof, when such substances as acetates were the source of carbon.

The investigation partially reported in this paper was started with the idea of determining the relation of the acid fastness of the tubercle bacillus to its power to produce immunity against infection with tuberculosis. Koch and many other workers have interested themselves in the question of an ideal tuberculosis vaccine and some recent workers have endeavored to rob the bacteria of waxy substances which are

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[CONTRIBUTION FROM THE KENT CHEMICAL LABORATORY, THE DEPARTMENT OF PATHOLOGY OF THE UNIVERSITY OF CHICAGO AND THE OTHO S. A. SPRAGUE MEMORIAL INSTITUTE.]

THE QUANTITATIVE DETERMINATION OF GOLD, ESPECIALLY IN ANIMAL TISSUE.[1]

BY SIDNEY M. CADWELL AND GLADYS LEAVELL.

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In connection with the experimental work carried on by Dr. Lydia M. DeWitt on the treatment of tuberculosis with various salts of gold, it was desired to determine the distribution of the gold in the organs of the guinea pigs used in the experiments. Since the dosage of gold was small, it was necessary to find a method which would determine accurately small fractions of a milligram of gold. Heubner[2] used a method for the determination of gold in animal tissue, but the amounts were always larger than those used in our experiments and only one test experiment is recorded.

The well-known assay method has been used for determining small amounts of gold, but was found unsatisfactory in our work because of the different class of impurities in our materials. Most of the published

[1] Part of the material in this paper has been embodied in a thesis presented to the Faculty of the University of Chicago by Sidney M. Cadwell in partial fulfillment of the requirements for the degree of Doctor of Philosophy, and part of the material will be used in a similar thesis by Gladys Leavell.

[2] *Arch. exp. Path. Pharm.*, **56**, 370.

methods for the quantitative separation and determination of gold depend on the great ease with which gold salts are reduced. In some cases the amount of reagent required to reduce the gold can be accurately determined. Utilizing this principle, Gooch and Morley have developed a method which is accurate to 0.005 mg. C. H. Christmann tried to apply this method in our problem but found that the ferric iron, as well as gold, oxidized the reagent. The possibility of the presence of other and varied oxidizing or reducing substances in the solutions form organic materials made it seem wise to choose a method which did not depend on oxidation by gold. The "Purple of Cassius" method, by which gold may be estimated in dilutions as great as one part to four million, was also found unsuitable for our purpose, since, unless the gold is first purified, the colors are not stable enough for comparison. Sarah Miller[1] has developed an electrolytic method for the separation of gold from ferric iron in phosphoric acid solution, but she was unable to deposit the last few milligrams of gold in the presence of iron until the voltage was raised to 2.7, at which point some copper is also deposited, if present. She also reports some difficulty with non-adherent deposits. Gold[2] has been electrolyzed from solutions containing it in the form of a complex cyanide, thioaurate, chloride and thiocyanide and also in the presence of free phosphoric acid. Neither potassium cyanide nor sodium sulfide solutions have been used for the electrolytic separation of gold from ferric iron because they precipitate the iron.

Of all the methods published the electrolytic method seemed most suitable for our work, but it was necessary to modify Miller's procedure, since we were working with much smaller amounts which would not be completely deposited by her method. Our gold, instead of being in the form of a chloride, was fulminating gold, formed in the course of dissolving the metal after the destruction of the organic matter. In our solution, also, some copper and iron were frequently, if not always, present. The conditions of the experiment require that the solution cannot be much more concentrated than one part of gold to 20,000 parts of water. Allamand states that the excellence of an electrolytic deposit is directly proportional to the concentration of the metal ions and inversely proportional to the current density within certain limits. To offset this large dilution, therefore, the rotating anode was used, thus maintaining the concentration immediately around the cathode; we thus not only improved the deposit, but also decreased the time required for deposition. The optimum temperature of about 60° and a current density of $N.D._{100} = 0.07$ were used. Our standard gold solution was prepared by dissolving

[1] This Journal, 26, 1268 (1904).

[2] Edgar F. Smith, "Electro Analysis," 5th ed., 1912; Allamand, "Electro Chemistry."

a known amount of pure gold in aqua regia. The excess of free chlorine was always removed by treatment with ammonia, thus forming fulminating gold before electrolysis.

In Table I, which summarizes the results of all the experiments made to test the method, the time is expressed in minutes and the weights in milligrams. The volume of the electrolyte was about 40 cc. and phosphoric acid and sodium phosphate were always added in the proportions prescribed by Miller. The temperature was 50 to 70° except in Expt. 1, when it was 34°. (See detailed description of method.)

TABLE I.

Summary of Test Experiments.

No.	Material analyzed.	Volts.	M. amp.	Time.	Au taken. Mg.	Au found. Mg.	Error. Mg.
1	Water	2.6	20	60	4.58	4.60	0.02
2	3 g. heart	1–1.5	2–8	105	2.28	2.26	0.02
3	5 g. kidney	1–1.5	2–10	105	2.28	2.45	0.17
4	10 g. liver	1–1.5	1–4	105	2.28	2.24	0.04
5	10 g. liver	1–1.5	2–7	105	2.28	2.29	0.01
6	$(NH_4)_2SO_4$	1.2	...	15	2.69	2.35	0.34
7	$(NH_4)_2SO_4$	1.2	...	30	2.69	2.72	0.03
8	$(NH_4)_2SO_4$	1.2	...	45	2.69	2.70	0.01
9	$(NH_4)_2SO_4$	1.2	...	60	2.69	2.79	0.10
10	$(NH_4)_2SO_4$	1.2	...	75	2.69	2.72	0.03
11	$(NH_4)_2SO_4$	1.2	...	90	2.69	2.74	0.05
12	8 g. spleen	1.2	...	10	2.69	1.89	0.80
13	4.5 g. kidney	1.2	...	20	2.69	2.43	0.26
14	7.31 g. lungs	1.2	...	30	2.69	2.68	0.01
15	19 g. liver	1.2	...	33	2.69	2.75	0.06
16	Feces	1.2	...	40	2.69	2.65	0.04
17	Urine	1.2	...	50	2.69	2.65	0.04
18	$(NH_4)_2SO_4$ 1.5 g. $(NH_4)Cl$ 1 g.	1.2	...	35	2.69	2.70	0.01
19	$(NH_4)_2SO_4$ 1.5 g. $(NH_4)Cl$ 1 g.	1.2	...	35	2.69	2.73	0.04
20	$(NH_4)_2SO_4$ 3 g. $(NH_4)Cl$ 2 g.	1.2	...	35	2.69	2.73	0.04
21	$(NH_4)_2SO_4$ 3 g. $(NH_4)Cl$ 2 g.	1.2	...	35	2.69	2.67	0.02
22	$(NH_4)_2SO_4$ 1.5 g. $(NH_4)Cl$ 1 g.	1.2	...	35	2.69	2.75	0.06
23	Copper 5 mg. Iron 5 mg.	1.2	...	35	2.69	2.72	0.03
24	$(NH_4)_2SO_4$ 1 g.[1]	1.1	...	40	2.69	2.73	0.04
25	$(NH_4)_2SO_4$ 1 g.[1] $(NH_4)Cl$	1.1	...	40	2.69	2.65	0.04
26	$(NH_4)_2SO_4$ 1 g.[2]	1.1	...	40	2.69	2.60	0.09
27	$(NH_4)_2SO_4$ 1 g.[2]	1.1	...	40	2.69	2.77	0.08
28	$(NH_4)_2SO_4$ 1 g.[3]	1.1	...	40	2.69	2.68	0.01
29	$(NH_4)_2SO_4$ 1 g.[3]	1.1	...	40	2.69	2.62	0.07

[1] Basic. [2] Neutral. [3] Acid.

TABLE I (*continued*).

No.	Material analyzed.	Volts.	M.-amp.	Time.	Au. taken. Mg.	Au. found. Mg.	Error. Mg.
30	5 g. liver	1–1.5	2–11	90	5.39	5.37	0.02
31	5 g. liver	1–1.5	2–14	90	5.39	5.43	0.04
32	4 g. liver	1–1.3	0.8–5	90	2.69	2.70	0.01 0.37%
33	10 g. liver	1–1.5	1.5–6	90	2.69	2.73	0.04 1.48%
34	10 g. liver	1–1.3	0.2–0.7	90	2.69	2.59	0.10 3.7%
35	10 g. liver	1–1.3	0.7–0.8	90	2.69	2.58	0.11 4.07%
36	10 g. liver	1–1.3	0.7–2	90	0.23	0.21	0.02 8.7%
37	10 g. liver	1–1.3	0.5–2	90	0.23	0.23	0.0 0.0%
38	10 g. liver	1–1.5	0.3–2	90	0.23	0.21	0.02 8.7%
39	10 g. liver	1–1.5	1.8–2	90	0.23	0.21	0.02 8.7%
40	15 g. liver	1–1.2	1.5–2	100	0.23	0.19	0.04 17.4%
41	15 g. liver	1–1.2	4–11	100	2.69	2.70	0.01 0.37%
42	10 g. liver	1–1.2	1.5–5	100	2.69	4.60	1.91 71.0%
43	$(NH_4)Cl$ $(NH_4)_2SO_4$	1–1.5	0.7–9	90	5.39	5.37	0.02 0.37%
44	$(NH_4)Cl$ $(NH_4)_2SO_4$	1–1.5	0.7–7	90	5.39	5.48	0.09 1.7%
45	10 g. liver	1.2	...	40	0.027	0.02	0.007
46	10 g. liver	1.2	...	40	0.054	0.06	0.006
47	10 g. liver	1.2	...	40	0.08	0.09	0.01
48	10 g. liver	1.2	...	40	0.11	0.12	0.01
49	10 g. liver	1.2	...	40	0.13	0.08	0.05
50	10 g. liver	1.2	...	40	0.00	0.01	0.01
51	$Au(CN)$	1.2	...	40	1.97	1.94	0.03
52	$Au(CN)$	1.2	...	40	1.97	1.95	0.02
53	$KAu(CN)_2$	1.2	...	40	2.61	2.60	0.01
54	$KAu(CN)_2$	1.2	...	40	2.61	2.66	0.05

Expts. 1 to 5 demonstrate that the method is applicable to our conditions. In Expts. 6 to 11, inclusive, the time of electrolysis was varied for a solution of gold chloride, ammonium chloride, ammonium sulfate and the usual phosphoric acid and sodium phosphate. In Expts. 12 to 17, inclusive, the gold chloride was added to a tissue solution and the time varied. Since fulminating gold was used in our actual experiments, we insured its formation in Expts. 18 to 23 by using the same

operations as in our regular analyses. Equivalent amounts of copper and iron were added in Expts. 22 and 23. In every case, as can be seen from the table, deposition was complete in 35 minutes.

In most of the experiments after deposition was complete the current was stopped and the electrodes were removed from the solution without siphoning off the electrolyte as is customary. To prove that this caused no appreciable error, two gold-plated electrodes were left in two phosphate solutions for two minutes, with losses of only 0.01 and 0.00 mg. In a similar experiment, 0.1 mg. was lost in a half-hour and only 0.25 mg. in 4.5 hours.

Since the solutions from organic materials may contain varying amounts of iron and copper, an experiment was run testing the deposition of iron from a tissue solution containing 0.01 g. pure ferric chloride. The method of analysis was the same as used with the gold and the gain in weight of the electrode was only 0.04 mg., an error easily accounted for by incomplete washing. In two experiments 0.05 g. copper sulfate was added to a tissue solution and an ammonium salt solution, respectively. No deposition occurred at 1.2 volts, but if the voltage was a few tenths higher the deposition was rapid; this copper deposit dissolved quickly, however, when the current was stopped.

To determine whether variations in acidity of the electrolyte caused variations in the result of the analyses, the solution in Expts. 24 and 25 had a strong odor of ammonia and was electrolyzed after the addition of one and one-half times the usual amount of sodium phosphate to make up for the absence of phosphoric acid. In Expts. 26 and 27, the solution was as nearly neutral as possible, no phosphric acid having been added. The solution in Expts. 28 and 29 was unusually acid, as 2 cc. of conc. hydrochloric acid was added after the usual preparatory steps were complete. As will be seen from the table, this varying basicity or acidity had very little effect on the deposition of the gold.

Having proved that gold can be determined quantitatively in solutions derived from the decomposition of tissues and that varying amounts of iron and copper do not interfere, 10 g. of guinea-pig liver containing gold was decomposed with sulfuric and nitric acids. As neutralization of the sulfuric acid loaded our solution unduly with salts, the sulfuric acid was evaporated from the Kjeldahl flask with the aid of a gentle current of air. Part of the gold was then in metallic form and was dissolved by the addition of aqua regia, the excess of free chlorine thus formed being removed by the addition of ammonium hydroxide. In Expts. 30 to 42 of Table I, gold was added to the tissue just after it was put into the Kjeldahl flask. The results show that small amounts of gold can be determined quantitatively in tissue by our method. In Expt. 42, the only unsatisfactory case, a precipitate was present in the electrolyzed solu-

tion and could be seen embedded in the deposited gold. Of the other experiments, only 34 and 35 show an error of more than 0.04 mg., this being a maximum error of 4% on the basis of 2.5 mg. gold. When only 0.23 mg. is determined, however, the variation of 0.04 mg. means a possible 20% error. The usual source of error is incomplete washing of flasks or electrodes, but with the use of the assay balance, a weighing error of 0.02 to 0.03 mg. may occur unless extraordinary precautions are observed. Expts. 43 and 44 were run to decide the effect of the presence of precipitates as in Expt. 42. There was a precipitate in Expt. 44 and none in Expt. 43. The results indicate that the electrolyte must be free from precipitate if reliable results are to be obtained. Filtration through an alundum plate which was packed in place of asbestos gave satisfactory results in the removal of insoluble residue in the electrolyte. In a few cases after several gold solutions had been filtered through the same filter, the residue and plate were treated with aqua regia and the resulting solution electrolyzed, but no gold was ever found. In Expts. 45 to 50 the solutions contained less than 0.02 mg. gold, since we had found that this was about the amount of gold which we might expect to find in our tissues. Since the animals received their gold as cyanides, Analyses 51 to 52 were run on aurous cyanide and 53–54 on potassium aurocyanide. The results show that gold in the form of cyanide can be determined by the method under consideration.

In one experiment a given amount of gold was injected into a living guinea pig which was then killed and analyzed, *in toto*. Only about 50% of the gold was recovered but the residue which had been filtered out was purple and the electrolyzed solution was yellow, both presumably colored by gold. Apparently, therefore, the method is not adequate to recover all the gold when a large amount of tissue is used. Since it had already been shown that the gold can be completely recovered from a 10 g. sample of tissue, the amounts recovered from a 20 and a 10 g. sample of the same tissue were compared as follows:[1]

	Gold found in 20 g. Mg.	Gold found in 10 g. Mg.
Sample No. 1	22	16
Sample No. 2	23	17
Sample No. 3	14	13
Sample No. 4	10	11

It is evident that all the gold is not recovered from a 20 g. sample.

An 8-hole Kjeldahl stand was used in the digestion of the tissue and 6 electrolyses were run at once. The average time required for complete

[1] It was noted that the amount of mineral matter in samples of tissue containing bone, in feces, and in urine was great and that results of analyses of these samples were less dependable than those obtained from analyses of other tissues. 5 g. samples gave better results in these analyses unless the amounts of gold present were too small.

analysis of each of about 250 samples was less than two hours. The report of this analytical work has been published by DeWitt, Cadwell and Leavell.[1]

Final Procedure.

The procedure in detail as finally worked out after all the preliminary tests had been made was as follows: 10 g. samples of fresh tissue[2] are placed in 300 cc. Kjeldahl flasks; 10 cc. of C. P. conc. sulfuric acid and 10 cc. of C. P. conc. nitric acid are added to it. The mixture is digested over a gas flame with the addition of nitric acid as needed, until the cooled sulfuric acid solution is colorless. Glass beads are inserted to prevent bumping during digestion. A glass tube through which clean air can be forced is run part way down the neck of the flask. The flask and its contents are then heated while the white fumes are blown out until the solution is concentrated to about 2 cc. If the solution is dark, nitric acid must be added to it and the mixture heated until it is colorless. When the solution is colorless, the tube is withdrawn and one cc. each of C. P. conc. nitric and hydrochloric acids are added. The mixture is boiled a few minutes, then one cc. more of conc. hydrochloric acid is added and the solution boiled again.

After the solution is cooled and diluted with 5 cc. of distilled water, C. P. conc. ammonium hydroxide is added until the color is discharged, then an excess of 2 cc. After the solution has been boiled one minute, there should still be an odor of ammonia at the mouth of the flask. If such is not the case, add more ammonia and boil again. More or less white precipitate often appears at this point. After the mixture has cooled, 5 cc. of conc. hydrochloric acid are added and the solution is boiled three minutes. Most of the non-crystalline precipitate should dissolve, but the boiling should not be continued more than three minutes. If any gold is present, the solution is usually yellow at this point. This solution is filtered by suction through an alundum plate packed with well-washed asbestos into the beaker in which the electrolysis is to be carried out. The Kjeldahl flask is rinsed through the filter with water acidulated with hydrochloric acid.

Ammonium hydroxide is added to the filtrate until the odor of ammonia is distinct. The gold usually does not precipitate at this time, but hydroxides of other metals may come down. The solution is made faintly acid and warmed if necessary to dissolve all the precipitate. To the solution,

[1] *J. Pharmacol.*, **11**, 357 (1918).

[2] If the tissue cannot be put directly into the Kjeldahl flask in which it is to be digested, it should be stored in small bottles and covered with 10 cc. of C. P. conc. sulfuric acid. The acid and undecomposed tissue can later be poured into a 300 cc. Kjeldahl flask. Any solid residue is stirred with C. P. conc. nitric acid and rinsed into the flask. The process is repeated with aqua regia to dissolve precipitated gold and then with water until the bottle is perfectly clean.

the volume of which should be about 40 cc., 1.1 cc. of 85% phosphoric acid and 0.75 g. of disodium hydrogen phosphate are added. It is then ready for electrolysis.

The electrodes are platinum. The cathode consists of a 1.5 cm. square plate of thin platinum foil to which is fused a platinum wire long enough to reach above the top of the beaker, and weighs less than 2 g. Any previous deposit of gold is first dissolved off with an approximately 3% solution of potassium cyanide and enough hydrogen peroxide to cause solution to take place quickly. Warming facilitates this reaction. After a thorough rinsing with water, nitric acid, water, alcohol, and ether, the electrode is dried and weighed.

During the electrolysis, the cathode is held below the level of the rotating anode by means of a bent glass rod. The temperature is kept at about 60°, but may vary from 50 to 70°. The electrode potential difference should never exceed 1.2 volts. The amperage may start as high as $N. D._{100} = 0.07$, but soon falls off considerably. After 40 minutes, the current is stopped and the cathode is withdrawn. It is very thoroughly washed with water then with alcohol and ether and dried at 140° for a few minutes, and weighed.

To demonstrate the correctness of this procedure, determinations were made of the percentage of the amount of gold injected into a living animal which could be recovered. Two guinea pigs were injected with a known amount of gold. They died a few hours later. Each body was ground, well mixed and duplicate 10 g. samples were analyzed. In one of the pigs 95% of the gold injected was accounted for and 100.6% in the other. In two other experiments, known amounts of gold were injected at intervals for two months into pigs which were kept in metabolism cages. By analyzing the excreta and samples of the bodies of the pigs, we were able to account for 92 and 97%, respectively, of the gold injected.

Determination of Larger Amounts of Gold in Inorganic Medium.—The above method having been proved satisfactory for the determination in organic materials of amounts of gold ranging from 2 mg. to 0.05 mg., it seemed desirable to test and, if necessary, modify the method so that it could be used for the determination of larger amounts of gold, in inorganic material, and for the separation of gold from iron and copper even when the latter are present in amounts nearly equal to that of gold.

This part of the investigation has been divided into 4 parts as follows: (1) the deposition of the gold as a bright adherent deposit from the fulminating gold solution; (2) the separation of gold from iron; (3) the separation of gold from copper; (4) the separation of gold from iron and copper.

The Precipitation of the Gold from Fulminating Gold Solution as a Bright Adherent Deposit.—As the solution of gold contained some free

chlorine, it was treated with ammonium hydroxide, etc., in the usual way.

In a preliminary experiment, 1.5 g. ammonium chloride was added to prevent the precipitation of gold. It was found that the deposition required 75 minutes. During the deposition there was some trouble with a precipitate and the final deposit was not sufficiently adherent. As this difficulty may have been due to the presence of too little ammonium chloride and too rapid precipitation of the gold, the experiments shown in Table II were run with varying amounts of ammonium chloride, a lower voltage and longer time. The voltage varied between 0.5 and 1.2, being kept at the lower figure for the first half hour; the current varied between 5 and 80 milliamperes. The time was 90 minutes.

TABLE II.

Deposition of Gold from Solutions with Varying Concentrations of Ammonium Chloride.

	Gold present. Mg.	NH_4Cl added. G.	Gold found. Mg.	Deposit.	Error. Mg.
1	31.5	3	31.4	Poor	0.1
2	31.5	3	31.1	Poor	0.4
3	31.5	6	31.2	Fair	0.3
4	31.5	6	31.2	Fair	0.3
5	31.5	9	31.4	Fair	0.1
6	31.5	9	31.6	Poor and solution spattered	0.1

The conditions of the third and fourth experiments seemed satisfactory and thereafter 6 g. of ammonium chloride was used in each electrolysis, and the voltage was kept at the lower value for at least the first thirty minutes.

The conclusions of the previous experiment were tested out in the series summarized in Table III. The voltage varied between 0.9 and 1.2 volts.

TABLE III.

Test Experiments on Deposition of Gold.

Gold present. Mg.	Gold found. Mg.	Error. Mg.	Remarks.
31.5	31.4	0.1	Good deposit
31.75	31.8	0.1	Good deposit
31.75	31.5	0.2	The deposit was not so good, as the rotating anode came too close to the cathode.

A final series summarized in Table IV was run in which it was the purpose to find the effect of varying the acidity. In this experiment the electrode potential difference was kept down to 0.5 volt for the first 50 minutes and at 1.1 volts for the last 40, making the total time for the deposition of the metal 90 minutes. The current varied from 0.35 to 0.02 ampere. In all cases the precipitates were good.

TABLE IV.

Deposition of Gold from Solutions with Varying Acidity.

Gold present. Mg.	Acidity.	Gold found. Mg.
31.75	slight as usual	31.6
31.75	slight as usual	31.6
31.75	2 cc. conc. hydrochloric in excess	31.6
31.75	2 cc. conc. hydrochloric in excess	31.65
31.75	large excess[1]	31.7
31.75	large excess[1]	31.8

The conclusion of these experiments is that gold can be determined satisfactorily from a fulminating gold solution according to the directions already given if to the solution of gold chloride containing from 0.03 to 0.04 g. of gold, 6. g of ammonium chloride is added to prevent precipitation, and the solution electrolyzed at 60° with a voltage of less than 0.6 volt for the first 30 to 45 minutes and below 1.3 for the remainder of the time. The deposition usually takes about 1.5 hours. It will be noted that the ammeter reading becomes very low at the end of the experiment, so can serve as an indication of the completion of the precipitation.

The Separation of Gold from Iron.—To determine whether gold could be deposited quantitatively in the presence of iron, one cc. of a ferric chloride solution containing about 0.025 g. of iron was added to the gold solution, and the experiment carried out as before. Throughout the experiment, the ammeter readings were greater, and the time for deposition was longer. Expts. 1 to 8 of Table V give the results.

The precipitates were dissolved off with potassium cyanide and hydrogen peroxide and were tested for iron with sodium thiocyanide, but no iron was found.

The Separation of Gold from Copper.—The experiments here were carried out just as in the case of the separation from iron, but one cc. of a solution of copper sulfate containing about 0.025 g. of copper was added to the solution of gold in each experiment. The results are shown in Table V, Expts. 9 to 13.

The deposits were all good. At the end of Expt. 10 the voltage became so high that a small amount of copper was deposited and this was removed by washing the electrode with dil. nitric acid before the final washing with water, alcohol, ether, and then drying.

The precipitates were dissolved off with potassium cyanide and hydrogen peroxide and tested for copper with ferrocyanide. No copper was present.

The Separation of Gold from Copper and Iron.—The experiments here were carried out just as in the cases of the copper and the iron separately,

[1] In these cases we first tried to electrolyze in an alkaline solution but the solution became cloudy and apparently conducted no current and several cc. of acid were added.

one cc. each of the iron and the copper solutions being added to each determination. The results are given in Table V, Expts. 14 to 18. The old deposits were all good.

TABLE V.

Deposition of Gold from Solutions Containing Iron and Copper.

	Gold present with iron. Mg.	Voltage. Volts.	Time. Hrs.	Gold found. Mg.	Error. Mg.
1	31.5[1]	0.6–1.2	2 hot 14 cold	31.5	..
2	31.75[2]	0.6–1.2	3 1/2	31.8	..
3	31.75	0.6–1.2	3 1/2	31.8	..
4	31.75	0.5–1.2	2 1/4	31.7	..
5	31.75	0.5–1.2	2 1/4	31.6	0.1
6	31.75	0.5–1.2	2 1/4	31.6	0.1
7	31.75	0.6–1.2	2	31.8	0.05
8	31.75	0.6–1.2	2	31.7	0.05
9	31.5	0.5–1.2	2 hot 10 cold	31.2	0.3
10	31.5	0.5–1.2	same as (9)	31.3	0.2
11	31.75	0.5–1.2	3 1/2	31.8	0.0
12	31.75	0.6–1.2	3 1/2	31.7	0.0
13	31.75	0.6–1.2	2 1/4	31.6	0.1
14	31.5[1]	0.7–1.1	1 1/2 hot 12 cold	31.3	0.2
15	31.75	0.5–1.2	3 1/2	31.7	0.0
16	31.75	0.5–1.2	3 1/2	31.7	0.0
17	31.75	0.6–1.2	2 1/4	31.7	0.0
18	31.75	0.6–1.2	2 1/4	31.5	0.2

Conclusions.

1. With a maximum error of 0.05 mg., 3 mg. or less of gold, present as fulminating gold, can be completely deposited electrolytically in 40 minutes from a phosphoric acid solution at a temperature of 60°, using a rotating anode and an electrode difference of potential of from 0.9 to 1.2 volts.

2. By the addition of 6 g. of ammonium chloride to prevent precipitation, 30 to 40 mg. of gold can be electrolyzed at a temperature of 60°, with a voltage of less than 0.6 volt for the first 30 to 45 minutes and below 1.3 volts for the remainder of the time, complete deposition usually requiring about 1.5 hours.

3. Under the conditions stated in (1) and (2), gold can be completely separated from equivalent amounts of copper and iron, but the time required is greater than if no copper and iron are present.

4. It makes very little difference whether the electrolyzed solution is neutral or much more acid than prescribed.

5. Using the method described, it has been possible to recover in 4

[1] Anode not rotated while solution was cold.

[2] The voltage was kept low for the first hour.

consecutive cases more than 90% of the gold injected into living animals.

6. The average time required for carrying out a complete analysis for gold by this method has been less than two hours.

Credit is due to Mr. L. M. Larson for some preliminary work done on this problem.

We take this opportunity of expressing our gratitude to Dr. Lydia M. DeWitt, of the University of Chicago, for her helpful guidance.

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THE RELATION OF PREGNANCY AND REPRODUCTION TO TUMOR GROWTH[1]

STUDIES IN THE INCIDENCE AND INHERITABILITY OF SPONTANEOUS TUMORS IN MICE

PROBLEMS IN THE BEHAVIOR OF TUMORS

TENTH REPORT

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Every biological fact concerning the behavior of cancer is a step toward the solution of the problems of that disease. Biological data help to form the very foundation of the most advanced medical science today. This is particularly true in regard to neoplasms, which have in many of their aspects, including heredity, been shown to conform closely to laws which are an established part of modern biology and which are no longer under dispute.

These biological data, then, cannot be brushed aside because we do not wish to believe them[2] or ignored because they interfere with some preconceived idea.

The literature of the experimental laboratory gives little data on the influence of pregnancy upon the rate of tumor growth. Leo Loeb (2) in his paper on Tissue Growth and Tumor Growth discusses in detail the general factors modifying tumor growth. In this paper he states that "in the rat, pregnancy seems to favor

[1] Delivered before the American Society for Cancer Research, Atlantic City, June 14, 1919.

[2] Ewing, J.: Neoplastic Diseases, Philadelphia, 1919, p. 107: "Nothing authorizes us to affirm that cancer is hereditary. In the interests of the public this doctrine ought to be combated."

growth of embryonal tissues under certain conditions; in the mouse it is unfavorable to such growth." Later in this same paper, he states "it will be necessary to distinguish more sharply than has been done in the past between the effect of pregnancy on the growth of *spontaneous* and of *transplanted* tumors." Whether the growths referred to above were spontaneous or transplanted is not stated. Woglom (3), reviewing this phase of cancer, said "It has been asserted and denied that the existence of pregnancy rendered animals less susceptible to *implantation*." Haaland found that pregnancy often exerted an inhibitory influence upon the proliferation of tumors, the effect of which was to produce a striking retardation of their growth in pregnant animals as compared with animals not bearing young.

Clinical literature yields case reports from which diverse conclusions are drawn. Bainbridge (1), reporting two of his own cases, states: "from these and other personal observations, as well as from the literature, it is apparent that pregnancy exercises a stimulating and hence a malign influence on coexistent cancer in any part of the body."

Bainbridge reviews the literature, and for purposes of ready reference I have added his bibliography to this article. He states:

> Siebold maintains he has observed a spontaneous cure of genital cancer owing to a supervening pregnancy. Pinard considers the rapid growth of cancer during pregnancy by no means proved, and Varnier reports a somewhat remarkable case: "In October 1897 the presence of an enormous carcinoma of the portio was ascertained in a pregnant woman. The following year there was again a pregnancy and death did not take place until October 1900."

Ewing (5) says: "Pregnancy has an unfavorable influence on the course of many uterine carcinomas, but this influence is not always apparent."

There is, therefore, in the light of these conflicting statements, need of exact data from the experimental laboratory on this subject, which in its fundamentals is entirely a biological subject, but whose clinical importance is very great.

This paper presents exact biological data concerning the behavior of spontaneous tumors in their relation to pregnancy and reproduction. In all this work nature is allowed to take her own course and is not interfered with in any way. In order to follow the most rigid exactions, I have confined this study to tumors of a specific type and of a specific organ, viz: alveolar tubular carcinoma of the mammary gland, some portions of some of the tumors being cystic. For purposes of comparison, a few other types have been introduced.

Five years ago I reported briefly on this point (4) and the subject has been under study ever since. The data which are here given represent, therefore, a portion of the accumulated evidence of five years and are perfectly typical of the behavior of tumor growth in its relation to pregnancy and reproduction.

In handling large numbers of these mice with spontaneous tumors, there is forced upon the observer from the very first (1) the tremendous difference in the rate of tumor grown in the non-reproducing and in the reproducing females; (2) the same tremendous difference in the amounts of tumor grown by a reproductive female during her reproductive and her non-reproductive periods.

The point is of such importance in the etiology of neoplasms as to warrant a very complete analysis of the facts.

For this demonstration I have selected thirty females not reproducing after the appearance of their tumors, but all of which had previously borne young; and thirty females constantly reproducing after the appearance of their tumors, all of which also had previously borne young. The data concerning the latter are divided into two periods, (1) the reproductive and (2) the non-reproductive periods. Charts 1 to 4 concern the females not reproducing after the appearance of their tumors, and give the following information on each mouse:

1. The duration of the tumor from the date found to the date of death.
2. The number of tumors.
3. The type of tumor.
4. Size in millimeters of the tumor when found.

5. Size of the tumor at death.
6. Causes of death.
7. Age of the mouse in days.
8. Total amount of tumor growth in cubic millimeters.
9. Average daily rate of tumor growth in cubic millimeters.

FEMALES NOT REPRODUCING AFTER THE APPEARANCE OF THEIR TUMORS

1. Female 6357 was picked up December 26, 1913, with a tumor in the right axilla 12 x 8 x 8 mm. or 768 cmm. She lived twenty-two days, during which time the first tumor became 30 x 30 x 28

CHART 1

No	Duration of Tumor	No. of Tumors	Type	Size in mm when found	Size in mm at Death	Cause of Death	Age	Rate of Tumor Growth [illegible] in Days	Average Daily Rate of Tumor Growth cmm
6357	[illegible] [illegible] 14 22 Days	3	(1) Rt Ax MG [illegible] Tub Carc [illegible] [illegible]	(1) 12x8x8 768 cmm (2) —— (3) ——	(1) 30x30x28 25200 cmm (2) [illegible] [illegible] cmm (3) [illegible]x10 1920 cmm	Tumor Gastric Hem	Dec [illegible] Jan [illegible] 14 390 Days	(1) 24432 (2) 360 (3) 1920 26712 in 22	1214.18
6754	[illegible] Mch 8 '14 [illegible] Days	3	[illegible] Hem [illegible] Tub Carc (2) Rt Fl [illegible] Small-Cell- Tub Carc (3) Lf Ax [illegible] Hem [illegible] Carc Tumor Embol [illegible]	(1) [illegible]x5x5 25[illegible] cmm (2) —— (3) ——	(1) 30x25x20 15000 cmm (2) 18x6x6 648 cmm (3) 6x6x6 216 cmm	Pulmonary Infect	Mch 14 13 Mch 8 14 356 Days	(1) 14750 (2) 648 (3) 216 15614 in 19	821.78
6933	Feb 15 '14 Mch 24 14 37 Days	4	(1) Rt Ax MG Alv Carc (2) Rt Sub Ax MG Hem Al Carc [illegible] (4) Rt Ing MG Cyst Ad Carc	(1) (2) 4x2x2 16 cmm (3) —— (4) ——	(1) 12x12x8 1152 cmm (2) 25x20x15 7500 cmm (3) 14x14x10 1960 cmm (4) 6x6x8 288 cmm	Tumors Chronic Nephritis	Apr 26 '12 Mch. 24 14 669 Days	(1) 1152 (2) 7484 (3) 1960 (4) 288 10884 in 37	294 16
7[illegible]25	[illegible] Apr 7 14 [illegible]	2	(1) Lf Ing MG Hem Carc (2) Rt Fl MG [illegible] Mult Metas Lungs	(1) 8x4x4 28 cmm (2) ——	(1) 40x30x30 36000 cmm (2) 8x6x6 288 cmm	Tumors Amyloidosis	[illegible] 13 Apr 7 '14 390 Days	(1) 35872 (2) 288 36160 in 37	977.29
7437	[illegible] 13 [illegible] 13 [illegible]	1	Ing MG Solid Med Carc	5x3x3 45 cmm	45x30x25 33750 cmm	Tumors Hyp Heart Term Infec	Sept 14 12 Sept 3 13 354 Days	33705 in 36	936 25
6[illegible]5	[illegible] Jan [illegible] 14 [illegible]	3	Rt Fl MG Alv Tub Carc [illegible] [illegible] Carc 3 Rt Ax MG [illegible]	(1) 6x3x2 36 cmm (2) —— (3) ——	(1) 30x26x20 15600 cmm (2) 3x3x3 27 cmm (3) 15x15x15 3375 cmm	Tumors Gastric Hem	Sept 14 12 Jan 26 14 488 Days	(1) 15564 (2) 27 (3) [illegible] 15594 [illegible] in 54	288.78
5561	Aug 29 Sept 24 13 26 Days	2	(1) Rt Ing MG Sq Cell Carc (2) Lf Lower [illegible] Adenoma [illegible]	(1) 10x6x6 360 cmm (2) ——	(1) 40x35x30 42 000 cmm (2) 2x2x2 8 cmm	Tumor Chronic Nephritis	Sept 28 12 Sept 24 13 361 Days	(1) 41640 (2) 8 41648 in 26	1601 84

mm. or 25,200 cmm. in size, while there also developed a second tumor of 360 cmm., and a third of 1920 cmm. She died January 17, 1914, of tumors and a gastric hemorrhage. She was then, at death, 1 year and 25 days, or 390 days old. During the period of tumor growth, 22 days, she grew 26,712 cmm. of tumor, an average of 1214.18 cmm. daily.

2. Female 6784, was found February 17, 1914, with a tumor 10 x 5 x 5 mm., or 250 cmm., in the left inguinal mammary gland. She died at 356 days of age, the original tumor having grown 14,750 cmm., while two other tumors of 648 cmm. and 216 cmm., respectively, had developed. She grew then 15,614 cmm. of tumor in 19 days, an average of 821.78 cmm. daily, dying of pulmonary infection.

3. Female 6933 died at 669 days old, of chronic nephritis, with a tumor growth of 10,884 cmm. in 37 days, an average of 294.16 cmm.

4. Female 7025 died at 396 days, of tumors and general amyloidosis, with a total tumor growth of 36,160 cmm. in 37 days, or an average growth of 977.29 cmm.

5. Female 5437 had a tumor of 45 cmm. when found; at death, 36 days later, the tumor measured 33,705 cmm., an average growth of 936.25 cmm. daily. She died when she was 354 days old of a terminal infection, with hypertrophied heart.

6. Female 6415, sister of 5437, died of tumors and a gastric hemorrhage at 488 days of age. She grew 15,594.37 cmm. of tumor in 54 days, an average of 288.78 cmm. daily.

7. Female 5581 was picked up August 29, 1913, with a right inguinal mammary gland tumor 10 x 6 x 6 mm. (360 cmm.). She died September 24, or 26 days later, of tumor and chronic nephritis, her tumor then measuring 42,000 cmm. She had grown 41,648 cmm. of tumor in 26 days, an average daily growth of 1601.84 cmm.

8. Female 5698 was found on September 15, 1913, with a tumor 8 x 5 x 4 mm. (160 cmm.) in the left inguinal mammary gland. She lived thirty days, during which time her first tumor grew 30,696 cmm. and she developed three other tumors of 2520, 125 and 240 cmm., respectively. She died at 352 days of age with terminal infection involving edema of the lungs and hydrothorax. She thus grew 33,581 cmm. of tumor in 30 days, an average of 1119.36 cmm.

9. Female 5723 on September 12, 1913, exhibited a left axillary mammary gland tumor 5 x 4 x 2 mm. or 40 cmm. She lived 36 days, growing 60,760 cmm. of tumor or an average of 1687.77 cmm.

At necropsy she disclosed a medullary carcinoma with multiple tumor emboli in the lungs.

10. Female 5753, sister of 5581, was found September 13, 1913, with a tumor of 240 cmm. in the right anterior mammary gland. She lived 27 days during which time her first tumor grew to be 45 x 45 x 30 mm. or 60,750 cmm., while a second tumor of 800 cmm. also developed. She grew then 61,310 cmm. of tumor in 27 days or an average of 2270.74 cmm. She died of her tumors at the age of 216 days.

CHART 2

No	Duration of Tumor	No of Tumors	Type	Size in MM when Found	Size in MM at Death	Cause of Death	Age	Rate of Tumor Growth CMM in Days	Average Daily Rate of Tumor Growth - CMM.
5698	Sept 15 - Oct 15 13 30 Days	4	(1) Lf. Ing M G Cyst Alv Carc (2) Lf Fl M G ditto (1) (3) Rt Fl M G ditto (1) (4) Ant to (3) ditto (1) Pul Metas	(1) 8 x 5 x 4 160 cmm (2) ——— (3) ——— (4) ———	(1) 38 x 28 x 29 30856 cmm (2) 18 x 14 x 10 2520 cmm (3) 5 x 5 x 5 125 cmm (4) 10 x 6 x 4 240 cmm	Tumors Edema of Lungs Hydrothorax Terminal Infection	Oct. 27. 12 Oct. 15. 13 352 Days	(1) 30696 (2) 2520 (3) 125 (4) 240 33581 in 30	1119.36
5723	Sept 12 - Oct 18 13 36 Days	1	Lf Ax M G Med Carc Tumor Emboli Lungs	5 x 4 x 2 40 cmm	40 x 40 x 38 60800 cmm	Tumor	Dec. 7 '12 Oct. 18 '13 325 Days	60760 in 36	1687.77
5753	Sept 13 Oct 20 '13 27 Days	2	(1) Rt Ant M G Cyst Hem Alv Carc (2) Rt Ax M G ditto (1)	(1) 8 x 6 x 5 240 cmm (2) ———	(1) 45 x 45 x 30 60750 cmm (2) 8 x 10 x 10 800 cmm.	Tumors	Mch. 18 '13 Oct 20' 13 216 Days	(1) 60510 (2) 800 61310 in 27	2270 74
6001	Oct. 9 - Nov. 23 '13 45 Days	3	(1) Rt Ax M.G Alv Tub Carc (2) Sub-Ax M G Cyst. Hem Carc (3) Lf. Ant M G Alv Carc Lf. Lower Lung Tumor Emboli	(1) 6 x 3 x 3 54 cmm (2) ——— (3) ———	(1) 22 x 20 x 10 4400 cmm (2) 15 x 13 x 10 1950 cmm (3) 4 x 4 x 4 64 cmm	Tumors Abscess in Liver. Gen. Infect.	June 2 '12 Nov. 23 '13 549 Days	(1) 4346 (2) 1950 (3) 64 6360 in 45	141.33
7247	Apr. 6 - May 4 14 28 Days	3	(1) Rt Ax M.G. Alv. Tub. Carc (2) Rt Ing M.G ditto (1) (3) Rt. Fl M.G. ditto (1)	(1) 4 x 2 x 2 16 cmm (2) ——— (3) ———	(1) 30 x 28 x 20 16800 cmm (2) 12 x 12 x 10 1440 cmm (3) 6 x 6 x 3 108 cmm.	Hyp. Heart Tumors	Apr 18 '13 May 4 '14 381 Days	(1) 16784 (2) 1440 (3) 108 18332 in 28	654.71
7324	Apr. 1 - May 14 14 43 Days	1	Lf Ant M G. Sq. Cell Carc	4 x 4 x 2 32 cmm	40 x 40 x 30 48000 cmm.	Tumor Term. Infect	Mch 12 '13 May 14 '14 428 Days	47968 in 43	1115.53
7467	Apr 20 - May 29 '14 39 Days	2	(1) Rt Ing. M.G Cyst. Alv Carc (2) Pelvic Alv Carc Rt Lung Riddled Metas	(1) 8 x 4 x 4 128 cmm (2) ———	(1) 43 x 30 x 30 38700 cmm (2) 20 x 18 x 15 5400 cmm	Tumors Amyloidosis	July 16 '13 May 29 '14 317 Days	(1) 38572 (2) 5400 43972 in 39	1127.48
7532	May 15 June 8 14 24 Days	1	Lf Ing M G Alv Tub Carc	6 x 3 x 2 36 cmm	30 x 25 x 25 18750 cmm	Tumor Term Infect.	Jan 25 '13 June 8 '14 489 Days	18714 in 24	779.75

11. Female 6001 was picked up on October 9, 1913, with a tumor 6 x 3 x 3 mm. (54 cmm.) in the right axilla. She lived 45 days after this date, her first tumor growing to 4400 cmm., along with two other tumors of 1950 cmm. and 64 cmm. respectively. She died at 549 days of age, necropsy showing an abscess in the liver with generalized infection and tumor emboli in the lung, in addition to the mammary gland tumors. She grew 6360 cmm. of tumor in 45 days, an average of 141.33 cmm.

12. Female 7247 was found on April 6, 1914, with a right axillary mammary gland tumor 4 x 2 x 2 mm. (16 cmm.). She lived 28 days thereafter during which time her first tumor became 30 x 28 x 20 mm. in size (16,800 cmm.). She grew also in this period a second tumor of 1440 cmm. and a third of 108 cmm. She died at 381 days of age, having grown 18,332 cmm. of tumor in 28 days, or an average of 654.71 cmm. daily.

CHART 3

No.	Duration of Tumor	No of Tumors	Type	Size in mm when found	Size in mm at death	Cause of Death	Age	Rate of Tumor Growth cmm in Days	Average Daily Rate of Tumor Growth cmm
7572	May 4 - June 16 '14 43 Days	6	(1) Lf Fl M G Tub Carc (2) Lf Ax M G Cyst Tub Carc (3) Lf Sub-Ax M G. Tub. Carc (4) Adjacent to (3) Hem Cyst Carc (5) Rt Ant M G Lymphoma (6) Lf Ant M G ditto (5)	(1) 4 x 4 x 3 48 cmm (2) 4 x 2 x 2 16 cmm (3) —— (4) —— (5) 4 x 4 x 4 64 cmm (6) ——	(1) 14 x 12 x 8 1344 cmm (2) 14 x 12 x 10 1680 cmm (3) 10 x 10 x 8 800 cmm (4) 5 x 5 x 5 125 cmm (5) 22 x 20 x 16 7040 cmm (6) 10 x 8 x 8 640 cmm	Tumors	Dec 5 '12 June 16 14 558 Days	(1) 1296 (2) 1664 (3) 800 (4) 125 (5) 6976 (6) 640 11501 in 43	26746
7761	May 25 July 8 '14 44 Days	1	Lf. Fl. M G Alv Tub Carc Lungs Riddled Metas	5 x 4 x 4 80 cmm	45 x 40 x 35 63000 cmm	Atrophied Organs from Tumor Pressure	July 19 13 July 8 14 354 Days	62920 in 44	1430.0
5357	July 15 - Aug. 25 '13 41 Days	1	Rt Ant M G. Tub Carc	4 x 4 x 4 64 cmm	40 x 30 x 25 30000 cmm	Hyp Heart Tumor	July 5 '12 Aug 25 '13 416 Days	29936 in 41	730.14
8042	July 5 - Aug 13 '14 39 Days	2	(1) Rt. Ax. M G Alv Tub Carc (2) Lf Ing M G ditto (1) Lungs Metas	(1) 5 x 3 x 3 45 cmm (2) ——	(1) 35 x 28 x 25 24500 cmm (2) 6 x 6 x 6 216 cmm	Tumors	June 30 13 Aug 13 14 409 Days	(1) 24455 (2) 216 24671 in 39	632.58
8104	July 1 - Aug 21 '14 51 Days	3	(1) Lf. Fl. M.G. Sq cell Carc Metas Reg Gland (2) Rt Ing. M G Alv Tub Carc (3) Lf Ing M G Sq. cell Carc Lungs Replaced Metas	(1) 6 x 5 x 3 90 cmm (2) —— (3) ——	(1) 45 x 40 x 35 63000 cmm (2) 2 x 2 x 2 8 cmm (3) 1.5 x 2 x 2 6 cmm	Tumors Terminal Infect	Oct 2 13 Aug 21 '14 323 Days	(1) 62910 (2) 8 (3) 6 62924 in 51	1233.80
8573	Sept 15' - Oct. 23 '14 38 Days	3	(1) Rt Fl. M.G Alv Tub Carc (2) Lf Ant M G ditto (1) (3) Lf Ax M G ditto (1)	(1) 5 x 4 x 2 40 cmm (2) —— (3) ——	(1) 40 x 30 x 30 36000 cmm (2) 3 x 3 x 3 27 cmm (3) 2 x 2 x 2 8 cmm	Tumors	Oct 28 '13 Oct 23 14 359 Days	(1) 35960 (2) 27 (3) 8 35995 in 38	947.23
8911	Oct. 29 - Dec 6 '14 38 Days	1	Rt Ax M G Alv Ad Carc Lungs Mult. Emboli	8 x 5 x 3 120 cmm	45 x 40 x 35 63000 cmm	Tumors	Apr 23 14 Dec 6 14 227 Days	62880 in 38	1654.73

A study of the charts will show parallel data concerning each of the other eighteen individuals in the class of mice which did not reproduce after the appearance of a tumor; in order to conserve space the other case reports are given in the charts only. The average daily rate of growth for the total number of these mice was 999.45 cmm. The youngest of these mice was 216 days, the oldest 803 days. The average age then, was 415 days or 1 year, 1 month, 21 days.

The second set of charts, nos. 5 to 8, deals with the reproducing females and gives the same items as the first set with the addition of the following:

10. The number of litters born after the appearance of tumor.
11. The total number of young after the appearance of tumor.
12. Time between the date of the last litter and the death date.

The total tumor growth and the average daily rate of growth are given in two groups: (1) the reproductive period, (2) the non-reproductive period.

CHART 4

No	Duration of Tumor	No of Tumors	Type	Size in mm when found	Size in mm at death	Cause of Death	Age	Rate of Tumor Growth cmm in Days	Average Daily Rate of Tumor Growth cmm
9365	Dec 2 14 Jan 6 15 35 Days	2	(1) Rt Ing M G Sq-cell Carc Lung Metas. (2) Pelvic Sq-cell Carc	(1) 10x10x6 600 cmm (2) ———	(1) 35x35x40 49000 cmm (2) 10x10x10 1000 cmm	Tumors Edema Lungs	Mch. 1 '14 Jan 6 15 311 Days	(1) 48400 (2) 1000 49400 in 35	1141.42
9510	Jan. 5 - Feb 24 15 50 Days	1	Lf Ax M G Alv Carc Lung Metas	6x2x2 24 cmm	45x40x40 72000 cmm	Tumor Chronic Nephritis	May 1 '14 Feb 24 '15 299 Days	71976 in 50	1439.52
1053	Feb 21 - Mch 14 12 22 Days	2	Ing M G (1) Sol Med Carc (2) Rt Ant. M.G Cyst Carc.	(1) 5x4x2 40 cmm (2) ———	(1) 35x22x17 13090 cmm (2) 8x5x5 200 cmm	Tumors Amyloidosis	Mch. 8 '11 Mch. 14 '12 371 Days	(1) 13050 (2) 200 13250 in 22	602.27
10244	Apr 18 May 18 '15 30 Days	1	Lf Ax M G. Alv. Tub. Carc	10x5x4 200 cmm	40x35x35 49000 cmm	Tumor Inflam Lungs	Sept 7 '14 May 18 15 253 Days	48800 in 30	1626.66
10253	Apr 1 - May 19 '15 48 Days	4	(1) Lf Ant M G Alv Tub Carc (2) Rt Ant M G ditto (1) (3) Rt Fl. M G ditto (1) (4) Rt Lower Lung Pap	(1) 3x3x2 18 cmm (2) ——— (3) ——— (4) ———	(1) 14x10x10 1400 cmm (2) 14x10x10 1400 cmm (3) 14x10x10 1400 cmm (4) 2x2x2 8 cmm.	Infected Tumors Terminal Infect	Mch 7 '13 May 19 '15 803 Days	(1) 1382 (2) 1400 (3) 1400 (4) 8 4190 in 48	87.29
10335	Apr. 21 - May 29 '15 38 Days	2	(1) Ant Dorsal Mid Line M G Tub Carc (2) Rt Ax M G ditto (1)	(1) 8x8x8 512 cmm (2) ———	(1) 45x35x35 55125 cmm (2) 3x3x3 27 cmm	Tumors Terminal Infect.	Aug 26 '14 May 29 15 272 Days	(1) 54613 (2) 27 54640 in 38	1437.89
19054	Jan. 17 - Feb 28 '18 42 Days	3	(1) Rt Ax M G. Alv Tub Carc (2) Rt Ant M G ditto (1) (3) Lf Fl M G ditto (1)	(1) 8x4x4 128 cmm (2) 4x2x2 16 cmm (3) ———	(1) 40x35x30 42000 cmm (2) 20x16x16 5120 cmm (3) 8x6x6 288 cmm	Tumors	Feb 1 '17 Feb 28 '18 392 Days	(1) 41872 (2) 5104 (3) 288 47264 in 42	1125.33
13565	June 1 - July 2 16 31 Days	4	(1) Lf Ax M G Pap Carc. (2) Lf Ing M G. Pap Ad Carc (3) Rt Ax M G. ditto (2) (4) Lf Lower Lung Carc Lung Metas	(1) 8x4x4 128 cmm (2) ——— (3) ——— (4) ———	(1) 36x25x20 18000 cmm (2) 8x6x6 288 cmm (3) 3x2x2 12 cmm (4) 8x6x6 288 cmm	Tumors	Jan. 4 '15 July 2 '16 544 Days	(1) 17872 (2) 288 (3) 12 (4) 288 18460 in 31	595.48

FEMALES CONSTANTLY REPRODUCING AFTER THE APPEARANCE OF TUMORS

1. Female 2426 was found June 16, 1912, with a tumor 10 x 5 x 5 mm. (250 cmm.) in the left inguinal mammary gland. She lived until September 7, 1912, or 83 days after the appearance of her tumor, during which time she produced 4 litters, with a total of 19 young. The last litter was born dead the day

CHARTS.

No.	Duration of Tumor	No. of Litters	No. of Young	No. of Tumors	Type.	Size in MM. When Found	Size in MM., At Death.	Cause of Death.	Time — Last Litter To Death	Age	Rate of Tumor Growth CMM in Days	Average Daily Rate of Tumor Growth CMM.
2426	June 16 — Sept. 7 '12 83 Days	4	19	1	Lf. Ing Tub. Carc. M.G.	10x5x5 250 Cmm.	15x15x15 3375 Cmm.	Hyp. Heart Thromb. Aur. Hydrothorax.	Sept. 6. (dead) Sept. 7 '12	June 27 '11 Sept. 7 '12 437 Days	3125 in 83	37.65 Reprod
3621	July 21 '12 Jan. 16 '13 179 Days	7	25	1	Rt. Ax. Alv. Tub Carc. M G	3x3x2 18 cmm Dec. 25 '12 8x3x2 48 cmm	16x8x8 1024 Cmm	Inf. with Liver Necrosis.	Dec. 25. '12 (dead) Jan. 16 '13	Dec. 5 '11 Jan. 16 '13 407 Days	30 in 157 976 in 22	.19 Reprod. 44.36 Non-Reprod.
4339	Sept. 15 '12 April 13 '13 210 Days	7	20	3	(1) Fl. to Fl. Sm cell Carc. M.G. (2) Rt. ant. Carc. M.G. (med-metas) (3) Lf. Lower Lung Pap Mult. Lung Metas	4x3x2 24 cmm Feb. 21 10x8x8 640 cmm	(1) 45x45x30 60750 Cmm. (2) 2x2x2 8 Cmm. (3) 4x4x4 64 Cmm.	Tumors	Feb. 21 (dead) April 13 '13	Sept. 10 '11 April 13 '13 580 Days.	(1) 616 in 159 60110 in 51 (2) 8 in 51 (3) 64 in 51 60182 in 51	3.87 Reprod. 1182. Non-Reprod.
4554	Jan. 30 — May 15 '13 105 Days	4	15	1	Rt. Ax Tub Carc M G	6x4x2 48 cmm April 30 6x6x6 216 cmm.	10x10x10 1000 cmm	Gen. Sepsis from Infected Uterus with Dead Foetus	April 30 (dead) - May 15 '13	Aug. 6 '12 May 15 '13 282 Days	168 in 90 784 in 15	1.86 Reprod 52.26 Non-Reprod
4751	Feb 13 — June 9 '13 116 Days	4	25	1	Rt Ax Alv Tub Carc M.G Mult. Small Metas. Lungs	10x4x3 120 cmm May 9 '13 10x8x8 640 cmm.	42x32x18 24192 cmm	Tumor	May 9. — June 9. '13	July 3 '12 June 9 '13 341 Days	520 in 85 23552 in 31	6.11 Reprod 759.74 Non-Reprod
5417	Oct 24 '12 Sept. 1 '13 311 Days	7	23	3	(1) Ing. M.G. Lar. Cell Alv Carc (2) Pelvic Lar. Cell Carc (3) Rt. Fl. M.G Tub Cyst. Carc Mult Lung Metas from (3)	(1) 8x5x5 200 cmm July 1 '13 10x8x6 480 cmm	(1) 52x42x28 61152 cmm. (2) 14x14x14 2744 cmm (3) 4x4x4 64 cmm	Tumors	July 1 (dead) Sept. 1. '13	April 30 '12 Sept. 1 '13 488 Days	(1) 280 in 250 60672 in 61 (2) 2744 in 61 (3) 64 in 61 63480 in 61	(1) 1.12 Reprod 994.62 Non- " (2) 44.98 " " (3) 1.04 " " 1040.64 Non-Rep.
5673	Dec. 31 '12 - Oct. 11 '13 284 Days	6	24	2	(1) Rt Ax. M.G. Tub. Cyst Carc (2) Lf Ing. M.G Tub Carc	(1) 8x5x5 200 cmm June 5 10x8x8 640 cmm	(1) 35x30x18 18900 cmm (2) 10x10x10 1000 cmm	Chr Neph	June 5 - Oct. 11 '13	Dec 16 '10 - Oct 11 '13 1024 Days	(1) 440 in 156 17360 in 128 (2) 1000 in 128 18360 in 128	(1) 2.82 Reprod 135.62 Non-Rep (2) 7.81 " " 143 43 Non Rep
6981	Oct. 1 '13 Mch. 31 '14 181 Days	6	30	1	Lf. Fl. M.G Alv Carc	6x5x4 120 cmm Feb. 20 '14 10x8x8 640 cmm	25x15x15 5625 cmm	Infected Tumor. Gen. Sepsis - Amyloidosis	Feb. 20 '14 (dead) Mch. 31 '14	Jan 30 '13 March 31 '14 425 Days	520 in 142 4985 in 39	2.95 Reprod 127.80 Non-Rep.
7246	Dec 19 '13 May 5 '14 137 Days	5	21	1	Rt to Lf Ax M G Alv Tub Carc Mult Lung Metas Leukemia	8x5x5 200 cmm. Apr. 4 '14 8x8x8 512 cmm	34x34x30 34680 cmm	Tumor and Leukemia	Apr. 4 — May 5 '14	June 2 '13 May 5 '14 337 Days	312 in 106 34168 in 31	2.94 Reprod 1102.19 Non-Rep.

before her death, so that she had no non-reproductive period after the appearance of her tumor. She died at 437 days of age, of a hypertrophied heart with thrombosis of the left auricle and hydrothorax. She grew 3125 cmm. of tumor in 83 days, an average of 37.65 cmm. daily during her reproductive period.

2. Female 3621 was found July 21, 1912 with a right axillary mammary gland tumor 3 x 3 x 2 mm. (18 cmm.). She lived 179 days after the appearance of her tumor. She produced 25 young in 7 litters during this period. The date of her last litter, born dead, was December 25, 1912, after which she lived 22 days. She had then 179 days of tumor growth during 157 of which she was reproducing; during the remaining 22 days she had no young. During her reproductive period she grew 30 cmm. of tumor in 157 days, an average daily growth of 0.19 cmm. During her non-reproductive period of 22 days she grew 976 cmm. of tumor, an average daily growth of 44.36 cmm.

3. Female 4339 was found September 15, 1912, with a tumor 4 x 3 x 2 mm. (24 cmm.) in the right flank mammary gland. She produced 20 young in 7 litters while growing her tumor. The last litter was born dead February 21, 1913. She lived 210 days after the appearance of her tumor during which time her first tumor grew to 45 x 45 x 30 mm. (60,750 cmm.) and a second and third tumor of 8 cmm. and 64 cmm. respectively developed. She died at the age of 580 days, having grown 616 cmm. of tumor in the 159 days she was reproductive, or an average of 3.87 cmm. daily. During her non-reproductive period of 51 days, she grew 60,182 cmm. of tumor, or a daily average of 1182 cmm.

4. Female 4554 was found January 30, 1913, with a tumor 6 x 4 x 2 mm. (48 cmm.), after which she produced 4 litters with a total of 15 young. Her last litter was delivered dead, on April 30, at which time her tumor measured 6 x 6 x 6 mm. (216 cmm.). She died May 15, at 282 days of age, of general sepsis from infected uterus with a dead undelivered fetus. At her death her tumor measured 10 x 10 x 10 mm. or 1000 cmm. She grew then during her reproductive period 1.86 cmm. daily; while during her non-reproductive period she grew 52.26 cmm. daily.

5. Female 4751 on February 13, 1913, showed a right axillary mammary gland tumor 10 x 4 x 3 mm. (120 cmm.). Between that date and May 9, she had 4 litters, a total of 25 young. On the date of her last litter her tumor measured 10 x 8 x 8 mm. (640 cmm.). Between May 9 and June 9, her tumor grew to 42 x 32 x 18 mm. (24,192 cmm.). She showed at necropsy multiple lung metastases. During her reproductive period, 85 days, she grew 520 cmm., an average of 6.11 cmm. daily. During her non-reproductive period, 31 days, she grew 23,552 cmm. of tumor, 759.74 cmm. average daily growth.

6. Female 5417 was found October 24, 1912, with an inguinal mammary gland tumor 8 x 5 x 5 mm. (200 cmm.). She bore 7 litters after this date, a total of 23 young, the last a litter of 1 born dead July 1, 1913. On this latter date her tumor was still only 10 x 8 x 6 mm. (480 cmm.). She lived until September 1, 1913, her tumor at death being 52 x 42 x 28 mm. (61,152 cmm.). She had also grown a pelvic tumor 14 x 14 x 14 mm. (2744 cmm.) and a right flank mammary gland tumor of 64 cmm. At necropsy her lungs showed multiple small metastases. During her reproductive period then, 250 days, she grew only 280 cmm. of tumor, an average of 1.12 cmm. daily. During her non-reproductive period of 61 days she grew 63,480 cmm., an average of 1040.64 cmm. daily.

7. Female 5673 was picked up December 31, 1912, with a right axillary mammary gland tumor 8 x 5 x 5 mm. (200 cmm.). She bore 6 litters, a total of 24 young, after this date. Her last litter was born June 5, 1913, at which date her tumor measured 10 x 8 x 8 mm. (640 cmm.). She died October 11, 1913, of chronic nephritis, being 1024 days old (2 years, 9 months, 24 days). She showed at necropsy a second tumor 10 x 10 x 10 mm. (1000 cmm.). During her reproductive period, 156 days, she grew 440 cmm. of tumor or an average of 2.82 cmm. daily, while during her non-reproductive period, 128 days, she grew 18,360 cmm. or 143.43 cmm. daily.

8. Female 7246 was found December 19, 1913, with a right axillary mammary gland tumor 8 x 5 x 5 mm. (200 cmm.). She bore 5 litters totalling 21 young after this date. Her last

CHART 6

No	Duration of Tumor	No of Litters	No of Young	No of Tumors	Type	Size in mm when Found	Size in mm at Death	Cause of Death	Time-Last Litter to Death	Age	Rate of Tumor Growth Cmm in Days	Average Daily Rate of Tumor Growth Cmm
7454	Sept 26 13 May 26 14 242 Days	6	20	1	Rt to Lf Ax M.G Sp Cell Sarcoma	10x10x10 1000 cmm. May 1'14 12x12x12 1728 cmm	42x40x30 50400 cmm	Tumor	May 1 - May 26 14	Feb. 21'13 May 26'14 459 Days	728 in 217 48672 in 25	3.35 Reprod 1946.88 Non-Rep
7536	Feb 20 June 9 14 109 Days	5	19	1	Lf Ax M.G Par Ad. Carc Lungs Riddled Metas	6x6x6 216 cmm May 22'14 8x6x6 288 cmm.	14x12x10 1680 cmm	Nematodes Tapeworm Typ. Changes	May 22 June 9'14	June 23'13 June 9'14 351 Days	72 in 91 1392 in 18	.79 Reprod 77.33 Non-Rep
7555	Jan. 13'14 June 13'14 151 Days	4	18	3	(1) Rt Ant M.G. Alv Tub Carc (2) Rt Ax M G Scirr Carc (3) Lf Ax. M.G Scirr Carc Mult Lung Metas.	(1) 8x8x8 512 cmm May 7'14 10x8x10 800 cmm	(1) 18x18x14 4536 cmm (2) 10x10x10 1000 cmm (3) 5x5x5 125 cmm	Hyp Heart. Lung Tumors	May 7 – June 13'14	Jan 19 13 June 13'14 510 Days	(1) 288 in 114 3736 in 37 (2) 1000 in - (3) 125 - - 4861 in 37	(1) 2.52 Reprod 100.97 Non-Rep. (2) 27 02 - (3) 3.37 - 131 36 Non Rep
8304	Mch 14 Sept 15'14 185 Days	4	17	1	Rt Ant M G Cyl cell Carc	4x4x4 64 cmm June 1'14 5x5x5 125 cmm	10x10x10 1000 cmm	Hyp Heart Hydro- thorax.	June 1 - Sept. 15'14	Sept. 5'12 Sept 15'14 740 Days	61 in 79 875 - 106	.77 Reprod 8.25 Non-Rep
8889	July 19 Dec 3'14 137 Days	5	20	4	(1) Lf to Rt Ax M.G 3 Nods Alv Tub pap Carc Sp. cell Sarc Tub Carc (2) Lf. Fl. M.G Early Cyst. Carc Lungs Riddled Metas	10x10x10 1000 cmm Nov. 19'14 20x20x20 8000 cmm	(1) 50x30x30 45000 cmm (2) 2x2x2 8 cmm	Tumors	Nov. 19. – Dec 3'14	Dec 7'13 Dec 3'14 392 Days	(1) 7000 in 123 37000 - 14 (2) 8 " " 37008 - 14	(1) 56.91 Reprod 2462 85 Non " (2) 57 " " 2643.42 Non-R.
9053	Sept 2 – Dec. 27'14 116 Days	4	18	1	Lf Ax M.G Sol Alv Carc.	6x6x6 216 cmm Dec 21'14 7x6x6 252 cmm	10x10x8 800 cmm	Tape worm Chr. Neph.	Dec 21. - Dec. 27'14	Mch. 9'14 Dec 27 14 293 Days	36. in 110 548. in 6	32 Reprod 91.33 Non-R.
9097	Nov. 5'14 Jan. 2 15 58 Days	3	10	1	Rt Ing M.G. Alv Tub Carc	10x10x10 1000 cmm Dec 20'14 10x10x10 1000 cmm	14x12x10 1680 cmm	Hem Left Ovary	Dec. 20'14 Jan 2'15	Mch. 12'13 Jan 2'15 661 Days	0. in 45 680 in 13	0. Reprod 52 30 Non-R

litter was born April 4, 1914, at which date her tumor measured only 8 x 8 x 8 mm. (512 cmm.). She died May 5, 1914, of tumors and leukemia, when she was 337 days old. She grew during her reproductive period, 106 days, 2.94 cmm. of tumor daily. During her non-reproductive period, 31 days, she grew 34,168 cmm. or 1102.19 cmm. daily.

9. The most striking illustration in this set of females is number 9172. She was picked up August 30, 1914, with a tumor 10 x 10 x 10 mm. (1000 cmm.) in the right axilla. After this date she bore 4 litters totalling 15 young. The date of her last litter was December 1, 1914, when her tumor measured only 11 x 11 x 10 mm. (1210 cmm.), having grown but 210 cmm. in 93 days or 2.25 cmm. daily. She lived 40 days longer and died January 10, 1915, of tumors, aged 337 days. During her non-reproductive period her tumor grew 98,790 cmm., while a left axillary carcinoma also developed to the size of 12 x 6 x 6 mm. (432 cmm.) making a total tumor growth of 99,222 cmm. in 40 days or 2480.55 cmm. daily (Chart 8).

10. Female 9097 is an interesting case. She was an old mouse, 661 days old when she died. She was found November 5, 1914, with a right inguinal mammary gland tumor 10 x 10 x 10 mm. (1000 cmm.). She bore 3 litters, 10 young, after this date. Her last litter was born December 20, 1914, at which date her tumor measured the same as when found, 45 days earlier. She lived 13 days longer, dying January 2, 1915, her tumor then being 14 x 12 x 10 mm. (1680 cmm.). She grew then 680 cmm. of tumor in 13 days while she was non-reproductive, an average of 52.3 cmm. daily. Necropsy showed a blood clot in the left ovary (Chart 8).

Charts 7 and 8 which cover the rest of the thirty reproducing females, show the same marked increase in tumor growth during the non-reproductive period. The average daily tumor growth for this set of mice was as follows:

1. During the reproductive period 7.75 cmm. daily.

2. During the non-reproductive period 686.34 cmm. daily, or over 88 times as much daily tumor growth as during the reproductive period.

CHART 7.

No	Duration of Tumor	No of Litters	No of Young	No of Tumors	Type	Size in MM when found	Size in MM at Death	Cause of Death	Time-Last Litter to Death.	Age	Rate of Tumor Growth Cmm. in Days	Average Daily Rate of Tumor Growth—
10015	Nov 21 '14 Apr 3 '15 133 Days	5	15	2	(1) Rt Ing. M.G. Alv Sarcoma Metas Lymph Gland (2) Rt. Ax. M.G. Partly Cyst. Sq. Partly Pap. Ad. Carc.	(1) 10x10x10 1000 cmm Mch. 21 '15 12x10x10 1200 cmm (2) ——	(1) 20x20x18 7200 cmm (2) 10x10x2 200 cmm	Liver Abscess Amyloidosis	Mch. 21 – Apr. 3 '15	Mch 11 '14 Apr. 3 '15 388 Days	(1) 200 in 120 6000 in 13 (2) 200 " " 6200 in 13	(1) 1.66 Reprod 461.53 Non-Rep (2) 15.38 " " 476.91 Non-Rep.
12189	Apr 1 '15 Jan 21 '16 295 Days	6	26	2	(1) Rt. Fl. M.G. Alv Tub. Cyst. Carc. (2) Lf Fl. M.G Ditto (1)	(1) 8x8x8 512 cmm Dec 10 '15 10x10x10 1000 cmm (2) ——	(1) 25x20x20 10000 cmm (2) 4x4x4 64 cmm	Chr. Neph	Dec. 10 '15 Jan. 21 '16	Apr. 26 '14 Jan. 21 '16 655 Days	(1) 488 in 253 9000 " 42 (2) 64 " " 9064 in 42	(1) 1.92 Reprod 214.28 Non-R. (2) 1.52 " " 215.80 Non-Rep
12852	Aug 18 '15 Apr 18 16 244 Days	8	31	3	(1) Lf. Ax M.G. Sol. Alv. Carc (2) Lf. Fl. M.G Cyst. Hem. Carc (3) Rt Mid. M.G Ditto (2)	(1) 8x8x8 512 cmm Mch 1 '16 12x10x8 960 cmm (2) —— (3) ——	(1) 30x25x25 18750 cmm (2) 20x18x18 6480 cmm (3) 10x6x6 360 cmm	Tumors	Mch. 1 '16 (Dead) Apr. 18 '16	Jan. 30 '15 Apr. 18 '16 443 Days	(1) 448 in 196 17790. " 48 (2) 6480 " " (3) 360 " " 24630 in 48	(1) 2.28 Reprod. 370.62 Non-Rep. (2) 135. " " (3) 7.5 " " 513.12 Non-Rep.
12963	Aug 3 15 May 3 16 274 Days	7	27	2	(1) Lf. Ant M.G. to Dor. Mid-line Alv Carc (2) Rt Mid-Lung Pap Carc	(1) 8x8x8 512 cmm Apr 1 '16 12x10x10 1200 cmm (2) ——	(1) 20x18x18 6480 cmm (2) 8x6x6 288 cmm	Hem. Ovary Lung Tumor	Apr 1 May 3 '16	Jan. 4 15 May 3 '16 485 Days	(1) 678 in 242 5280 " 32 (2) 288 " " 5568 " "	(1) 2.80 Reprod 161.87 Non-R (2) 9. " " 170.87 Non-R
15650	Mch 15 16 " 4 17 354 days	6	22	2	(1) Rt. Ing. M.G. Hem. Alv Tub Carc (2) Rt Ant. M.G Ditto (1)	(1) 4x4x4 64 cmm Feb. 5 '17 10x8x5 400 cmm (2) ——	(1) 20x20x20 8000 cmm (2) 8x8x8 512 cmm	Intest. Infect.	Feb 5 – Mch 4 17	Aug 12 '15 Mch. 4 '17 569 Days	(1) 334 in 327 7600 " 27 (2) 512 " " 8112 in 27	(1) 1.02 Reprod 281.48 Non-Rep. (2) 18.96 299.44 Non-Rep.
16046	Nov 16 16 Apr 18 17 153 Days	5	17	2	(1) Rt. Ing M.G Tub Alv Carc (2) Rt Sub-ax. M.G (Ditto (1))	(1) 4x4x4 64 cmm Mch 11 '17 8x8x8 512 cmm (2) Mch. 27 '17 10x10x10 1000 cmm	(1) 18x16x16 4608 cmm (2) 11x11x11 1331 cmm	Chr. Neph.	Mch. 27 – Apr 18 '17	Nov. 25 '15 Apr. 18 '17 509 Days	(1) 448 in 131 (2) 1000 in 131 1448 in 131 (1) 4096 in 22 (2) 331 " " 4427 in 22	(1) 3.41 Reprod (2) 7.63 " 11.04 Reprod (1) 186.18 Non-Rep (2) 15.04 " " 201.22 Non-Rep.
15990	Dec. 13 '16 Apr 12 '17 120 Days	3	13	1	Lf Ax. M.G Hem Cyst Carc.	8x8x8 512 cmm Feb 28 '12 12x12x12 1728 cmm	40x40x40 64000 cmm	Tumor Pul. Inf with Hem.	Feb. 28 (Dead) Apr. 12 '17	Apr 30 '16 Apr 12 '17 348 Days	1216 in 77 62272 in 43	15.79 Reprod 1448.18 Non-Rep

CHART 8

No.	Duration of Tumor	No. of Litters	No. of Young	No. of Tumors	Type	Size in MM When Found.	Size in MM at Death	Cause of Death	Time-Last Litter to Death	Age	Rate of Tumor Growth-Cmm in Days	Average Daily Rate of Tumor Growth Cmm
16371	Dec. 18 '16 May 22 '17 155 days	3	7	2	(1) Rt. Ax. M.G Alv Tub. Carc (2) Lft. Ax. M.G. Ditto (1)	(1) 8x8x8 512 Cmm Feb. 28 10x10x10 1000 Cmm (2) ———	(1) 35x35x30 36750 Cmm (2) 10x10x10 1000 Cmm	Hydrothorax Inf. Areas Lung	Feb 28 '17 May 22 '17	May 1 '16 May 22 '17 387 Days	(1) 488 in 72 35750 in 83 (2) 1000 in 83 36750 in 83	(1) 6 77 Reprod 430.72 Non-Rep (2) 12.04 - 442.76 Non-Rep
18845	Feb 19 '17 Feb. 1 '18 347 days	6	32	2	(1) Rt. Ing. M.G. Alv Carc (2) Rt. Ant. M.G. to Spine Ditto (1) Every Lobe Lung nearly Re- placed-Metas	(1) 4x4x4 64 Cmm Dec. 9 '17 8x8x8 512 Cmm (2) Nov 25 '17 6x6x6 216 Cmm	(1) 25x20x18 9000 Cmm (2) 25x25x25 15625 Cmm	Tumors Acute Neph	Nov 25 '17 Feb 1 '18	July 5 '16 Feb 1 '18 576 Days	(1) 448 in 279 (2) 216 in 279 664 in 279 (1) 8488 in 68 15409 in 68 23897 in 68	(1) 1.60 Reprod (2) .77 " 2.37 Reprod (1) 124.82 Non-Rep. (2) 226.60 " " 351.42 Non-Rep.
17466	May 16 '17 Aug. 30 '17 106 days	5	13	1	Rt Ax. M G Alv Tub Carc	4x4x4 64 Cmm Aug 14 '17 8x8x8 512 Cmm	18x18x18 5832 Cmm	Chr Neph.	Aug. 14- Aug 30 '17	Oct. 1 '16 Aug. 30 '17 333 Days	448 in 90 5320 in 16	4.97 Reprod 332.50 Non Rep.
15768	(1) Oct. 23 '16 Mch. 19 '17 (2) Feb. 27 '17 Mch. 19 '17 (3) T. Eye Sept. 29 '16 Mch. 19 '17 147 days	4	18	3	(1) Lft Ing M.G. Cyst-Alv. Tub.- Carc (2) Lft Sub. Ax. M.G. Alv. Tub Carc. (3) Lft. Lower Lung nearly Replaced-Pap	(1) 8x8x8 512 Cmm Feb. 27 '17 12x12x12 1728 Cmm (2) 10x10x10 1000 Cmm (3) ———	(1) 25x25x25 15625 Cmm (2) 25x20x20 10000 Cmm (3) 10x10x10 1000 Cmm	Tumors Chr Neph	Feb. 27 (dead) March 19 '17	Apr 1 '16 Mch 19 '17 352 Days	(1) 1216 in 127 13897 in 20 (2) 9000 in 20 (3) 1000 in 20 23897 in 20	(1) 9.57 Reprod 694.85 Non-Rep (2) 450 00 " " (3) 50.00 " " 1194 85 Non-Rep.
9172	Aug 30 '14 Jan 10 '15 133 days	4	15	2	(1) Rt Ax. M G Alv. Carc. (2) Lf Ax M G Pap Carc Lungs Riddled Metas	(1) 10x10x10 1000 Cmm Dec 1 '14 11x11x10 1210 Cmm (2) ———	(1) 50x50x40 100000 Cmm (2) 12x6x6 432 Cmm	Tumors	Dec. 1 '14 Jan 10 '15	Feb 7 '14 Jan. 10 '15 337 days	(1) 210 in 93 98790 in 40 (2) 432 in - 99222 in 40	(1) 2.25 Reprod 2469.75 Non-Reprod (2) 10 8 2480 55 Non. Reprod
9712	Jan 5 '15 Apl 28 '15 113 days	4	18	1	Lf Ax. M G to Base Ear Hem Ad Carc	10x10x10 1000 Cmm Apr 10 '15 20x15x12 3600 Cmm	40x40x35 56000 Cmm	Tumor	Apr 10- Apr 28 '15	June 10 14 Apr 28 '15 322 days	2600 in 95 52400 in 18	27 36 Reprod 2911 11 Non Reprod
9996	Oct 24 '14 Mch 31 15 158 days	5	24	1	Rt Sub-Ax MG Tub Pap Carc Lungs Replaced Metas	8x8x8 512 Cmm Feb 11 '15 10x8x8 640 Cmm	20x15x20 6000 Cmm	Lung Tumors Retained Fetus Gen. Sepsis	Feb 11- Mch. 31 '15	July 7 '13 Mch 31 '15 632 days	128 in 110 5360 in 48	1 16 Reprod 111.66 Non-Reprod

The youngest of these mice was 282 days, the oldest was 1024 days, the average age was 465 days or 1 year, 3 months, 10 days, the majority of the mice being in the height of the reproductive age at the time of the origin of their tumors. Every mouse from the youngest to the oldest shows a tremendous increase of tumor growth after she ceased to be reproductive.

Note the three cases of mammary gland sarcoma which have been introduced here for purposes of comparison.

1. Female 7454, who had at death a spindle-cell sarcoma right to left axilla 42 x 40 x 30 mm. (50,400 cmm.), grew only 3.35 cmm. of tumor daily during her reproductive period of 217 days after the appearance of tumor, while she grew an average of 1946.88 cmm. of tumor daily in the 25 days she was non-reproductive (Chart 6).

2. Female 8889 had at death a tri-nodular tumor 50 x 30 x 30 mm. (45,000 cmm.), this being a spindle-cell sarcoma between two alveolar tubular carcinomas, the whole growth extending from the left to the right axilla and down to the right forefoot. This mouse showed an average daily tumor growth of 56.91 cmm. during her reproductive period of 123 days, while the rate of growth jumped to an average daily rate of 2643.42 cmm. during the fourteen days she lived after she ceased reproducing (Chart 6).

3. Female 10,015 with a right inguinal alveolar sarcoma and a right axillary partly cystic-squamous, partly papillary adenocarcinoma yielded the following data: The sarcoma grew an average of 1.66 cmm. daily during the 120 days she was reproducing, while an average daily growth of 461.53 cmm. succeeded in the 13 days she was non-reproductive. The carcinoma developed entirely during her non-reproductive period at the daily rate of 15.38 cmm. (Chart 7).

The papillary adenocarcinomas showed the same relative rates of growth during the reproductive and the non-reproductive periods. Note female 7536, (chart 6), average daily growth 0.79 cmm. while reproductive, 77.33 cm. while non-reproductive.

The problem of this retardation of tumor growth during the period of active reproduction is a complex one, and it is necessary to eliminate the other obvious factors in such delay.

Let me repeat at this point what I already have published frequently, viz: the mice in this laboratory have behind them many generations of hygiene as perfect as the most rigid care can secure. They come of strains whose members are vigorous, sleek, active, well grown, long-lived, and highly reproductive. The individuals taken for this study are among the most vigorous mice in the laboratory. A glance at the number of litters and the number of young they bore after the appearance of their tumors will attest this vigor to anyone who has ever tried the work of breeding mice with spontaneous tumors. These mice have been kept as free from other diseases as it is possible to keep them. Nevertheless many of the cases are complicated by causes of death other than tumor, and this is obviously one of the factors in the varying amount of tumor grown by different individuals. The relation of other diseases to the amount of tumor growth is a subject much too large for the confines of this paper. It will be treated in detail in a forthcoming communication; but let me at this time point out a few facts in this connection as shown by the individuals listed in these charts.

Female 3621 (chart 5) died of an infection characterized by extensive liver necrosis. She lived 22 days after the birth of her last litter (born dead) and grew during this time only 976 cmm. of tumor, 44.36 cmm. daily average. She was 1 year, 1 month, 12 days old, scarcely past the prime of reproductive life in mice. Compare this amount of tumor growth with that of female 8889 (chart 6) who was just 15 days younger and who died of tumors uncomplicated by any other disease. This latter grew 37,008 cmm. of tumor in the 14 days she lived after her last litter, an average daily growth of 2643.42 cmm.

Female 4554 (chart 5) died May 15, 1913, of general sepsis from infected uterus with an undelivered fetus remaining from her last litter, born dead April 30. She was 282 days (9 months, 12 days) old, in the prime of reproductive life. She grew only 784 cmm. of tumor in the 15 days she lived after her last litter, or an average daily growth of 52.26 cmm.

Compare this amount of tumor growth with that of female 9172 (chart 8), fifty-five days older, where the cause of death was not complicated by any other disease than tumor. This latter female grew 99,222 cmm. in the 40 days she lived after the birth of her last litter, an average daily growth of 2480.55 cmm.

CHART 9

Non-Reproducing Females —		
Age in Days	Average Daily Tumor Growth cmm	Cause of Death
216	2270.74	Tumor
227	1654.73	Tumor
253	1626.66	Inflam. Lungs
272	1437.89	Term. Infect.
299	1439.52	Chronic Neph.
311	1141.42	Edema Lungs
317	1127.48	Amyloidosis
323	1233.80	Term. Infect.
325	1687.77	Tumors
352	1119.36	Term. Infect. Pulmon Infect
354	1430.	Atrophied Organs From Tumor Pressure
354	937.50	-Term. Infect.
356	821.78	Pulmon. Infect.
359	947.23	Tumor
361	1601.34	Chronic Neph.
371	602.27	Amyloidosis
381	65471	Hypertrophied Heart
390	1214.18	Gastric Hem.
392	1125.33	Tumors
396	977.29	Chronic Neph.
409	632.58	Tumors
416	730.14	Hypertrophied Heart
428	1115.53	Term. Infect.
488	288.78	Gastric Hem.
489	779.75	Term. Infect
544	595.48	Tumors
549	141.33	Abscess in Liver Term. Infect.
558	267.46	Tumors
669	294.16	Chronic Neph.
803	87.29	Term. Infect.

Female 9053 (chart 6), only 293 days old, died of tapeworm. She grew only 548 cmm. of tumor in the 6 days she lived after her last litter, an average daily growth rate of 91.33 cmm.; while female 7536 (chart 6), who died of tapeworm and nematodes, grew an average of only 77.33 cmm. of tumor in the 18 days she lived after her last litter. She was 351 days old.

Compare these two with female 15768 (chart 8), 352 days old, who had only a slight chronic nephritis in addition to her tumors. She produced a daily average of 1194.85 cmm. of tumor in the 20 days she lived after her last litter (born dead); compare also with female 15,990 (chart 7) of about the same age, 348 days, who had a brief acute pulmonary infection. She produced an average of 1448.18 cmm. of tumor daily.

In every tumorous mouse that I have handled whose disease was complicated by tapeworm or nematodes or both, tumor growth has been seriously interfered with, as it has been seriously interfered with by any other rapidly destructive type of complicating disease.

Other diseases, then, are one factor modifying the rate of tumor growth.

CHART 10

Tumors Only	
Age in Days	Daily Rate Tumor Growth cmm
216	2270.74
227	1654.73
325	1687.77
359	947.23
392	1125.33
409	632.58
544	595.48
558	267.46

Age, as is rather generally admitted, is evidently another factor which modifies the rate of tumor growth.

I have drawn up a chart in age sequence, of the thirty females not reproducing after the appearance of their tumors (Chart 9).

The youngest mouse here, 216 days old, showed the largest average rate of tumor growth, viz: 2270.74 cmm. daily, while the oldest mouse, 803 days, produced the smallest daily average of tumor growth, viz: 87.29 cmm. The intermediate mice show a fairly well graduated decrease in average daily tumor growth as the age increases, the relatively few irregularities being explained at least in part by the complicating diseases. For if we take those that died of tumors only, shown in chart 10, the sequence is pretty regular from the youngest, 216 days old and with a daily rate of 2270.74 cmm., to the oldest, 558 days old with a daily rate of 267.46 cmm.

For those dying of a complicating pulmonary infection, the sequence is entirely regular, the youngest, 253 days, growing 1626.66 cmm. daily, while the oldest, 356 days, grew 821.78 cmm. daily.

Of those dying of a terminal infection the sequence again is notably regular from the youngest, 272 days, with an average

CHART 11

Pulmonary Infection	
Age in Days	Daily Rate Tumor Growth
253	1626.66 cmm
311	1141.42
352	1119.36
356	821.78

CHART 12

Terminal Infection	
Age in Days	Daily Rate Tumor Growth
272	1437.89 cmm
323	1233.80
352	1119 36
354	937.50
428	1115.53
489	779.75
549	141.33
803	87 29

growth of 1437.89 cmm. to the oldest, 803 days, with an average rate of 87.29 cmm.

For those dying of chronic nephritis the sequence is fairly regular, and for those dying of gastric hemorrhage, entirely so.

CHART 13

Chronic Nephritis	
Age in Days	Daily Rate Tumor Growth
299	1437.89 cmm
361	1601.84
396	977.29
669	294.16

CHART 14

Gastric Hemorrhage	
Age in Days	Daily Rate Tumor Growth
390	1214.18 cmm
488	288.78

In the non-reproducing females, then, there appear only two complicating factors outside of the general metabolic condition of the mouse and the fact that it is at the height of the reproductive age but is not reproducing, viz: the age of the mouse, and those diseases other than tumor which cause death.

Bearing in mind, then, that both age and a destructive complicating disease modify the results, the tremendous amount of tumor uniformly grown by these non-reproducing mice (of reproductive age) stands out with extreme clearness. The normal

course of spontaneous tumors in mice of reproductive age which are not being bred, is very rapid. The shortest duration of tumor growth in these mice was 19 days; the longest was 54

CHART 15

REPRODUCING FEMALES			
AGE IN DAYS.	AVERAGE DAILY TUMOR GROWTH WHILE REPRODUCTIVE C MM	AVERAGE DAILY TUMOR GROWTH WHILE NON-REPRODUCTIVE C MM	CAUSE OF DEATH
282	1.86	52.26	INFECTED UTERUS (DEAD FETUS) GEN SEPSIS
293	.32	91.33	TAPEWORM CHR NEPH
322	27.36	291 11	TUMOR
333	4.97	332 50	CHR NEPH.
337	2.25	2480.55	TUMOR
337	2.94	1102.19	LEUKEMIA.
341	6 11	759.74	TUMOR
348	15 79	1488.18	PUL. INFECT WITH HEM
351	.79	77.33	NEMATODES TAPEWORM
352	9.57	1194.85	CHR NEPH
387	6.77	442.76	HYDROTHORAX PUL. INFECT
388	1.66	476.91	LIVER ABSCESS AMYLOIDOSIS
392	56 91	2643.42	TUMOR
407	.19	44.36	LIVER NECROSIS
425	2.95	127.80	TERM INFECT
437	37.65		HYP HEART HYDROTHORAX
443	2 28	513 12	TUMOR
459	3.35	1946 88	TUMOR
485	2 80	170.87	HEM. OVARY LUNG TUMOR
488	1 12	1040 64	TUMOR
509	3.41	201 22	CHR. NEPH
510	2.52	131.36	HYP. HEART LUNG TUMORS
569	1.02	299.44	INTEST. INFECT
576	2.21	351.42	ACUTE NEPH.
580	3 87	1178 62	TUMOR
632	1.16	111.66	RETAINED FETUS GEN SEPSIS
655	1 92	215.80	CHR NEPH
661	.0	52 30	HEM OVARY
740	.77	8.25	HYP. HEART HYDROTHORAX
1024	2 82	143.43	CHR NEPH

days; the average was 35 days, or 1 month, 5 days. These tumors grow to a great size, frequently being as large as the body of the mouse itself. In over 70 per cent of the cases multiple tumors arose, there being but 8 cases with one tumor only; 8 cases with two tumors; 9 cases with three tumors; 4

cases with four tumors; and one case with six tumors. 13 cases, or nearly one-half, showed pulmonary metastases.

In the age chart of the reproducing females the influence of age upon the amount of tumor growth is much less apparent (Chart 15). Even when charted in age periods of 100 days, the influence of age upon tumor growth is much less evident in the reproducing females than in the non-reproducing (Chart 16).

This is in part explained by the complicating causes of death, such as tapeworm and nematodes in the digestive tract, general sepsis from an infected uterus with dead retained fetus, etc. For if we take the females dying of carcinoma only, eliminating

CHART 16

AVERAGE DAILY GROWTH cmm.			
	NON-REPROD.	REPRODUCTIVE	
AGE PERIODS —		NON-REPROD. PERIOD	REPROD. PERIOD
200-300 DAYS	1685.70	71.79	1.09
300-400 DAYS	1108.13	1053.59	12.28
400-500 DAYS	709.35	640.11	7.19
500-600 DAYS	334.75	435.21	2.60
600-700 DAYS	294.16	126.58	.69
700-800 DAYS		8.25	.77
800-900 DAYS	87.29		
1000 DAYS		143.43	2.82

CHART 17

REPRODUCING FEMALES. DYING OF CARCINOMA ONLY		
AGE IN DAYS	TUMOR GROWTH WHILE REPRODUCING	TUMOR GROWTH WHILE NOT REPRODUCING
322	27.36 cmm	2911.11 cmm
337	2.25	2480.55
341	6.11	759.74
443	2.28	513.12
488	1.12	1040.65
580	3.87	1182.00

those that died of more rapidly growing sarcoma, or of complicating destructive diseases like tapeworm and general sepsis, we get a better sequence (Chart 17).

Even here, however, there is evidence of *some other factor affecting the amount of tumor grown by reproducing females.*

If we chart these same carcinomatous females in sequence of the number of young borne while they were tumorous, we get almost a perfect sequence, the one exception apparently being accounted for by age difference. In these reproducing females, then, *the number of young borne after they are tumorous, seems also to be a factor in determining the amount and rate of tumor growth.*

Bearing in mind these three factors, age, other complicating diseases, and the number of young borne, note the tremendous increase of tumor growth in these mice after they cease reproducing.

The normal course of these spontaneous mammary gland tumors in mice which are constantly reproducing, is very slow. In many cases the tumor scarcely grows at all during this period, one tumor showed no growth whatever during 45 days of reproduction. The lowest daily rate of tumor growth was 0.19 cmm.; the highest daily rate was 56.91 cmm.; the average rate was 7.75 cmm. The duration of the tumor is greatly prolonged by reproduction, the mouse sometimes living nearly a year after the appearance of her tumor while she bears many litters of young. The shortest duration of tumors in these mice was 58 days, the longest 347. The average duration was 177.87 days or 5 months, 27.9 days as compared with 1 month, 6.4 days in the non-reproducing females of about the same age.

CHART 18

Reproducing Females Dying of Carcinoma Only			
No. of Litters	No. of Young	Rate of Tumor Growth while not reproducing	Age in Days
4	15	2480.55 cmm	337
4	18	2911.11	322
7	20	1182.00	580
7	23	1040.65	488
4	25	759.74	341
8	31	513.12	443

The smallest number of young borne by these mice after their tumor appeared was 7, the largest number was 32; the average was 20.

When these females cease reproducing the tumors grow with great rapidity, the mouse frequently living only a few days after the birth of her last litter. The shortest period after the last litter was less than 1 day, the longest was 128 days (in a mouse 2 years, 9 months, 24 days old). The average duration of the tumor in the non-reproductive period was 37 days.

This average duration period is almost exactly the period of duration of the tumor (35 days) in the thirty non-reproductive females of almost the exact age, which therefore make a valuable control in this study of the reproducing females. During this brief period, often only 8 or 10 days, the tumor grows to many

times the size attained during the entire period of reproduction, averaging nearly six months.

The lowest daily rate of tumor growth during the non-reproductive period was 8.25 cmm. (in a mouse nearly 2 years old). The highest daily rate was 2911.11 cmm. (in a very young mouse about 10 months old); the average rate was 686.34 cmm., considerably less than the average daily rate of the non-reproducing females of nearly the exact average age (999.45 cmm.) whose tumors were of nearly the exact average duration (35 days).

This emphasizes again the probability that the number of young borne after the appearance of the tumor is a factor in the amount of tumor grown after the reproductive period is past,

CHART 19

Averages		
	Non-Reproductive ♀	Reproductive
Age	415 Days (1 yr. 1 mo. 20 days)	465 Days (1 yr. 3 mo. 10 days)
Duration of Tumor	1 mo. 6.4 Days	5 mo. 27.9 Days
Daily Rate of Tumor Growth cmm	999.42	686.34 Non-Reprod Period 7.75 Reproductive Period

and links these two modes of growth, the growth of embryos and the growth of tumor in a very close relationship. Multiple tumors appeared much less frequently in these reproducing females than in the non-reproducing. There were 15 cases of single tumor growth, as compared with 8 cases in the non-reproductive females. There were 9 cases with two tumors, as compared with 8 in the non-reproducing females, 5 cases of 3 tumors as compared with 4 in the non-reproducing, while 4 was the highest number of tumors occurring in any one of these reproducing mice, there being but one such case. 50 per cent of these cases showed multiple tumors as compared with over 70 per cent of multiple tumors in the non-reproducing females. Ten cases, or one third, showed lung metastases.

Chart 19 shows the average age, duration of tumor growth, and rate of tumor growth of the 2 sets of mice, the non-reproducing and the reproducing.

SUMMARY

In handling large numbers of mice with spontaneous tumors there is forced upon the observer from the very first the great difference in the rate of tumor growth in the non-reproducing and in the reproducing females.

The same difference is noted in the rate of tumor growth in the non-reproductive and in the reproductive periods of the same female.

For this study thirty each of non-reproducing and of reproducing females with spontaneous tumors were selected. The tumors were all of the same type and of the same organ (with a few exceptions for purposes of comparison), viz: alveolar tubular carcinoma of the mammary gland, of which a daily observation is easily made.

Without exception, the amount of tumor grown by a female while reproductive was strikingly less than during her non-reproductive period.

Again, the amount of tumor grown by reproducing females was strikingly less than that grown by non-reproducing females.

The normal course of these spontaneous tumors in mice that are not bred is very rapid, the mouse rarely living over six weeks and often less than a month after the appearance of her tumor. The tumors grow to a great size, frequently being as large as the body of the mouse.

When, however, these tumorous mice are bred, the tumor scarcely grows at all during the reproductive period. The duration of the tumor is greatly prolonged, the mice frequently living nearly a year after the appearance of their tumors, during which time many bear from six to eight litters aggregating from twenty to thirty-two young. When the mouse ceases reproducing, the tumors grow with tremendous rapidity and to great size, the female frequently surviving only six or eight days after the birth of the last litter. During this brief period the tumor grows to many times its size at the date of the last litter.

In brief, during the six or eight days a mouse is non-reproductive, she grows enormously more tumor than during the eight months or a year while she is reproductive, the daily rate of

tumor growth being far in excess of the daily rate during the reproductive period.

Two other factors must be taken into consideration, viz: the age of the mouse, and other complicating causes of death. Generally speaking, the younger mice show a higher daily rate of tumor growth than do the older mice.

Again, complicating diseases such as tapeworm and nematodes in the digestive tract, or any other highly destructive disease, greatly retard tumor growth; and *the number of young borne after the appearance of tumor is also a factor in reproducing females.* But when these factors have been eliminated two facts stand out with startling clarity and cannot be gainsaid, viz:

1. Reproducing females grow much less tumor than do non-reproducing females of the same approximate age and general metabolic condition.

2. Reproducing females grow much less tumor while they are reproductive than they do while they are non-reproductive; in other words, *when a mouse is producing embryos, she is not producing tumor in anything like the amount which she grows while non-reproductive.* Multiple tumors are more common in the non-reproducing than in the reproducing females, the figures being over 70 per cent compared with 50 per cent.

In striking contrast to these results is the relation between pregnancy and the infections common to mice. If an infected mouse is bred, instead of having the infection held off for a year or more while she bears young, she is unable to produce any young at all and speedily dies of her infection. Or if a pregnant mouse contracts an infection, she rarely brings her young to birth.

The results of this study bring out with striking force the close relation between tumor production and the production of young, showing them to be two closely related modes of growth.

CONCLUSIONS

1. Cancer and reproduction, both being growth processes, draw upon the same energy residuum and are made possible by the same food. Hence the food and energy used by one are withheld from the other.

2. Therefore (a) if the female is *constantly pregnant*, energy and food are withheld from the tumor and it grows with extreme slowness. (b) If there is a hiatus between pregnancies, or a termination of pregnancy, the energy which was running into reproduction is released and diverted into tumor which grows very rapidly. (c) If tumor growth considerably antedates impregnation, the currents of energy are already being used for tumor growth and are with difficulty diverted for pregnancy, probably never wholly so.

3. Hence, when a female is well advanced in tumor growth before impregnation there are rarely any offspring brought to birth. When offspring are delivered they are few, small, undernourished, and rarely suckled (which in mice means there is no lactation).

4. When tumor growth is not interfered with by pregnancy, it is (a) extremely rapid in mice which are young, well nourished, and vigorous; (b) less rapid in mice older or less vigorous, or less nourished; (c) very slow in mice which are old, feeble, under nourished, or afflicted with a destructive complicating disease.

5. Another point which shows the close relation between the growth of embryos and the growth of tumor is the great frequency with which breast tumors are nearly synchronous with delivery. Hyperstimulation of any tissue seems to originate cancer of those tissues in individuals of cancer tendency; hence the intense stimulation incident upon lactation tends to originate cancer of the breast in individuals of breast cancer tendency.[3]

6. The prolonged hiatus between pregnancies greatly complicates the study of the relation between pregnancy and tumor growth in the human species. During this prolonged hiatus the tumor may draw off the energy which would have continued to be used in reproduction if the pregnancies were not widely separated, just as is the case in mice kept constantly impregnated. This would account for any apparently conflicting testimony in human experience as compared with these studies.

[3] This subject will be more fully treated in a forthcoming paper.

The factors are not subject to control in the attempt to study the relation between reproduction and tumor growth in the human species, and the conclusions have to be drawn without knowledge of complicating factors. The real relation between these two can be disclosed only in the experimental laboratory, where the factors are all known and are under control.

The experimental evidence shows a very striking relation between these two modes of growth, the production of young and the production of tumor; moreover, it shows the same relation between the production of young, and the growth of *all types* of mammary gland tumors.

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PRIMARY SPONTANEOUS TUMORS OF THE TESTICLE AND SEMINAL VESICLE IN MICE AND OTHER ANIMALS

XII. STUDIES IN THE INCIDENCE AND INHERITABILITY OF SPONTANEOUS TUMORS IN MICE

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Tumors of the testicle would seem to be uncommon in mice, for we have been unable to find even a single case reported in the literature, although they have been described in other species of lower animals. Thus, among 103 primary tumors observed by McCoy (1) among 100,000 rats killed in plague work, there was one described as an "angiosarcoma" of the testicle, without further details. Other series of tumors in wild rats (Woolley and Wherry (2), Beatti (3)) do not record cases of testicular tumors. Caspar (4) says:

> Carcinomas of the testicle, often described in horses and dogs, form sometimes soft, sometimes hard tumors, in which not rarely single portions are formed differently. Through mucoid and colloid degeneration of the cell nests, cysts with gelatinous contents may be formed.

Infiltration along the spermatic cord and lymph-node metastases are often observed. Horses appear especially to develop testicular tumors, particularly if we consider the relatively small number of old animals that have not been castrated. In Japan, where this operation is less often performed than in Europe, equine testicular tumors are most abundant. Thus Kimura records the finding of 49 such growths among 142 tumors observed in 77,224 slaughtered horses. This may be compared

with the figures given in the census statistics on the mortality from cancer in the registration area of the United States, which shows that of 52,420 cases of malignant tumor there were but 121 recorded as arising in the testicles, or 2.3 per thousand; as 21,282 of the cancer cases were males, the proportion of testicle tumors is 5.8 per thousand of all tumors in males. Kimura studied in detail 12 specimens of equine "orchidoblastomas" varying in size up to 7500 grams weight; all were unilateral, and in at least five cases there were metastases in the spermatic cord and the inguinal and lumbar lymph-nodes. He describes their anatomic characteristics as follows:

1. Tumors usually develop without altering the normal shape of the testis. The albuginea is somewhat thickened, showing more or less engorgement of blood-vessels on its surface and expanding with the growth of the tumor, and may continue to enclose it even when of large size.

2. Generally the entire glandular portion of the organ is replaced by new growth, but sometimes the atrophied deep brownish glandular portion may remain under considerable compression at the peripheral part.

3. The tumor consists of nodules, which vary considerably in size and are generally surrounded and separated by strands of fibrous tissue in varying amount.

4. On section the cut surface of the tumor parenchyma is of medullary yellowish gray-white colour and of soft or somewhat firm consistence, whilst here and there creamy yellowish red-gray islets which represent the softened necrotic areas, and various-sized irregular deep red hemorrhages can be seen, so that these figures give an almost marble-like appearance.

5. The spermatic cord and the inguinal and lumbar lymph-nodes are frequently apt to become involved, and distant metastases perhaps may occur (dissemination in the peritoneal cavity).

Microscopical examination

1. Corresponding with the macroscopic appearance, bands of fibrous tissue are seen traversing the section in all directions. These vary in width and divide the parenchyma into islets of various sizes and shapes, giving an alveolar appearance.

2. The tumor cells are roughly polyhedral, spheroidal, or ellipsoidal in shape, the cytoplasm stains in general feebly with eosin and appears homogeneous or slightly granular, varying in size from 11 μ to 35 μ in diameter.

The nuclei are of three kinds, according to their shape, size, and staining qualities.

(i) Spheroidal or short ellipsoidal, deeply stained small nuclei with invisible nucleoli.

(ii) Ellipsoidal large nuclei with vacuolar appearance, in which only the nuclear membrane and nucleolus have taken on the stain, and the latter stains bright red with eosin (usually only one, sometimes two or three in number).

(iii) Nuclei which show intermediate characters between (i) and (ii).

The size of these nuclei varies from 6.5–16 μ in diameter. Multinuclear cells with strikingly large cytoplasm and a circular arrangement of their nuclei about a central lumen are numerous in all cases. Mitotic figures are abundant in the tumor cells, most of them being bipolar, though figures with three, four, or more poles, and cells with irregular distribution of chromosomes are not infrequently met with. Generally the tumor cells are rich in glycogen, but only a small amount of fat is to be seen; there are small areas of haemorrhage and necrosis in the tumor parenchyma, and the fat can be shown circumscribed especially in the latter, in sections stained with Sudan III.

3. Traversing the section in all directions and cutting up the parenchyma into islets, the fibrous stroma gives off finer strands—sometimes only a few fibers—running in towards the peripheral portion of the enclosed parenchyma from the main circumferential band; but the tumor cells lie along the walls of the alveoli without intimate connection with them and are packed together without fibrillar intercellular substance, so that not all the tumor cells are intermingled with the fibrous stroma. The stroma represents more or less round-cell infiltration, which mostly consists of lymphocytes; and reactive proliferation of the connective tissues may sometimes be seen. Elastic fibers appear generally in small amount.

4. In the atrophied residual glandular portion, spermatogenesis is usually not visible; only one case among four with surviving glandular tissue showed spermatogeny. The cells in the atrophic seminiferous tubules greatly resemble tumor cells morphologically. Interstitial tissue and membrana propria of the seminiferous tubules are in general

increased, and masses of granular cells in the interstitium (*Zwischenzellen*) appear atrophic.

As compared with these observations in Japan, Sticker (6), in his compilation of the European literature on the occurrence of tumors in the lower mammals, reports that of 298 malignant tumors in horses, but 11 were in the testicle: in cattle, of 110 tumors none were in the testicle, and there were none among the recorded neoplasms of sheep (7 cases), of cats (7 cases), or of swine (7 cases). But of 766 tumors in dogs, 18 were in the testicle. Of 305 cases of bovine tumor observed in the slaughter house at Glasgow, according to Trotter (7), there was none in the testicle. Undoubtedly these figures give an entirely false impression as to the frequency of testicular tumors in the lower animals, since so few old, uncastrated horses, cattle, swine, or sheep come to the slaughter houses, where most of the neoplasms are detected. According to Caspar (4) a testicular cancer has been observed in a cat by Leiserling, metastases having been found in the mesentery of the transverse colon. A case of carcinoma of the bovine testicle is mentioned without details by Murray (8), and Williams (9) says that in the ass sarcoma of the testicle has been described; he also mentions the occurrence of tumors in the testis of swine, but without citing the origin of these reports. Wolff (10), in his compilation of the literature, refers to several cases of testicular tumor in dogs and horses, but records none in mice or other animals. He refers to the case, reported by Axe, of a tumor arising in an ectopic testicle in a pony, and several other cases in the literature suggest that in dogs and horses, as in man, the ectopic testicle is especially likely to undergo malignant transformation.

One of us (H. G. W.) has observed two cases of primary tumor of the testicle in dogs, which, because of the infrequency of recorded cases in the literature, are briefly described here, as follows:

Case I. A pure blooded Pomeranian, age five and one-half years, was brought to the laboratory by Dr. J. W. Walker, in order that a growth involving the right testicle might be removed by operation.

This growth had been noticed at least two years before and had grown very slowly. The operation was done under anesthesia; the animal recovered and is now in apparently perfect health three years after the operation. No metastases could be palpated in the groin or elsewhere. The animal had been suffering from extensive loss of hair for some time before the operation, but after it the normal coat was restored. The growth was about 3 x 2 x 2 cm., involving the body of the testicle as an encapsulated nodule and leaving a compressed remnant of testicle tissue at one side. There was no infiltration, and the epididymis was not involved.

Microscopically it shows irregular coarse lobules with heavy fibrous tissue trabeculae, packed full of large cells with little cytoplasm and pale vesicular nucleus closely resembling the spermatogonia. The capsule is very dense and contains a few islands of tissue cells. Despite the heavy fibrous tissue the cells do not seem to be compressed. In the residual testicle tissue there is some spermatogenesis going on. This seems to represent an unusually benign, slow-growing type of the usual large cell tumor of the testicle.

Case II. A mongrel fox terrier dog, apparently moderately old, was killed in the course of experimental work in the Department of Physiology of the University of Chicago, and an autopsy was performed by Dr. A. B. Luckhardt. The right testicle, which was within the scrotum, was replaced by a tumor about 6 cm. in diameter, entirely contained within the tunica albuginea, and not adherent to the surrounding tissues. No metastases could be found in the regional nodes or elsewhere. The prostate, however, was swollen, and irregular in shape and consistence, and contained a purulent fluid. The left testicle lay within the inguinal canal and was decreased in size to about one-half the normal dimensions, although the epididymis was apparently normal. Nothing is known of the history of this animal, but it was striking in that the teats resembled those of a bitch that has nursed repeatedly.

Microscopically the tumor resembles the usual type of alveolar carcinoma seen in human cases (fig. 1). The cells are large, consisting chiefly of a large vesicular nucleus; the stroma is heavy and coarse, and divides the tumor not only into alveoli but also into lobules; only in a few areas is it cicatricial. Mitoses are abundant. A striking peculiarity in this tumor is the tendency of the cells in some alveoli to form long cords parallel to each other and at right angles to the basement membrane, producing an unusual effect of palisade arrangement.

Sections through many parts of the tumor show no residue of testicle tissue, and no teratomatous elements. The prostate shows an extensive round cell interstitial infiltration, with numerous polymorphonuclear leucocytes within the tubules. The atrophied ectopic testicle shows an epididymis resembling that of the preadolescent testicle, while the testicle itself consists of ill-defined tubules with ordinarily but a single row of irregular cells near a thickened basement membrane, although the center of the tubules contains much protoplasmic debris which is

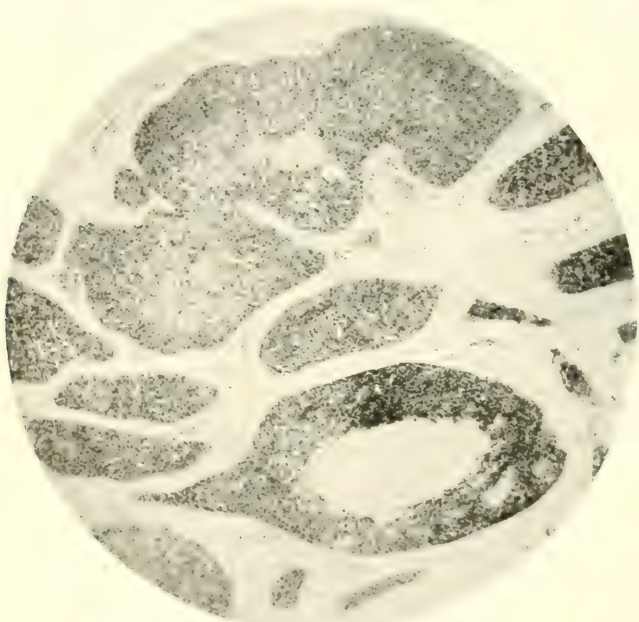

FIG. 1. PRIMARY CARCINOMA OF THE TESTICLE OF A DOG. (× 80)

often pigmented. The stroma is moderately increased, but Leydig's cells are not conspicuous. Several of the tubules are full of large deeply staining cells resembling those of the tumor of the opposite testicle. These give the impression that in these isolated atrophic tubules neoplastic proliferation is beginning to arise independently.

In other species of animals, although castration is not commonly practiced, testicle tumors are rare. The reviews of the literature on spontaneous tumors of birds and fowls by Joest

and Ernesti (11), and by Pentimalli (12), mention only three testicular tumors. One was a teratoma replacing the testicle of a chicken, described by Winokuroff (13) and the other two were cases of sarcoma of the testicle in parrakeets (*Palaenoris Eupatrius?*), reported by Fox (14). One of these was bilaterally symmetrical, probably derived from the testicle, and described as a large round cell alveolar sarcoma; the other was unilateral, and undoubtedly arose in the testicle. In his extensive autopsy experience with wild animals Fox has found no case of tumor of the testicle, nor could we find any such cases in the literature, unless we include the instance recorded by Pick and Poll (15) of a cystoma testis with carcinomatous areas and a secondary nodule, which occurred in the testicle of a Japanese giant salamander (*Kryptobranchus japonicus*).

From such descriptions of the histological findings of the testicular tumors of lower animals as are found in the literature, and which are mostly meagre, these growths seem to present the same characteristics as those observed in man. The prevailing type is those tumors of large polyhedral cells in alveolar arrangement, which are often described as alveolar sarcoma or, when the normal relation of the tumor cells to the nutritive vessels is conspicuous, angiosarcoma. Lymphoid infiltration of the stroma is also commonly described in animals as well as in human tumors. Ewing (16), in his critical review of testicular neoplasms, says of the human material: "The commonest tumor of the testis is an embryonal carcinoma, alveolar or diffuse, with polyhedral or rounded cells and often with lymphoid stroma." This statement holds equally well for the testicular growths of animals as described in the literature. Ewing also states: "These tumors are probably one sided developments of teratomata," an origin which is not suggested by those authors who describe animal tumors, although Caspar (4) quotes Kitt as saying that chondromas are found repeatedly in the testes of horses; on the other hand, Kimura does not even mention the occurrence in horses of tumors suggesting a teratomatous character. Furthermore, Frank (17), in his discussion of the histogenetic origin of testicle tumors, concludes that the typical

large cell tumors are not derived from teratoid tumors, but from spermatogonia. Kimura, in his study of equine material, also came to the conclusion that these tumors are derived from the epithelium of the seminiferous tubules.

TESTICULAR TUMORS IN MICE

In 19,000 autopsies on mice of the Slye stock we have found 28 tumors arising in the testicle. These figures, it should be emphasized, apply to mice living a natural life and reaching as great an age as it has been possible to make then attain by the best of care and the most rigorous hygienic precautions; all died natural deaths, without having undergone any experimental interventions or manipulation. Of the 19,000 about one-half were males. Statistics have not yet been compiled as to the total number of tumors arising in male mice, although they are much less common than in females, most abundant being tumors of the lung, subcutaneous tumors, sarcomas, tumors of the testis, and adenoma of the liver. As we have pointed out in previous papers, the great predominance of females among tumor mice, observed and emphasized by other writers, depends solely upon the frequency of mammary tumors. Excluding these and other sex gland tumors, we find an approximately equal distribution. Thus, in our published lung tumor series (18) of 160 cases there were 42.6 per cent in males, and 57.4 per cent in females; in 28 liver tumors (19) there were 50 per cent in each sex; in 8 cases of cancer of the stomach (20) also, equal numbers were observed in each sex. Sarcomas are more abundant in female mice than in males in the ratio of 2 to 1 because the mammary gland is a common site of sarcomas (21). The tumors of the testicle offset in large measure the tumors arising in the ovary.

That we have obtained so large a number of tumors of the testicle is explained by the fact that all these tumors, with one exception (7308), occurred in the mice in a single strain, No. 90, and its hybrid derivatives. This exceptional case was not a typical testicular growth, but a spindle cell sarcoma. As has

been emphasized in previous papers, not only the tendency to develop cancer depends on the ancestry of the mice, but also the localization of tumors in special organs or tissues (see Slye (22)). Had not this particular strain been developed, hybridized extensively, and followed for several years, there would have been but a single case of testicle tumor to record, and that a sarcoma. We may mention the fact that some forms of growth observed in other laboratories seem to be relatively infrequent in the Slye stock, e.g., the keratinizing type of lung tumors described by Tyzzer and Haaland, and the preputial gland adenomas observed especially in Bashford's laboratory. As material accumulates this tendency of certain tumors to occur in certain strains of mice becomes more and more distinct; this has its counterpart in human pathology—e.g., the uterine fibroids of the negro, and the repeatedly described predominance of tumors of certain viscera in certain families.

Most of these new growths of the testis are essentially benign in character, developing very slowly, rarely ulcerating, generally distinctly limited by the tunica albuginea, and in no case with distinct remote metastases (fig. 2). Some cases have remained under observation for as long as eight months, and in only a few instances have they seemed to be the cause of death, through urinary retention or ascending suppurative nephritis (5 cases), local necrosis and suppuration (3 cases), or hemorrhage (3 cases). They have varied in size from about 5 mm. in diameter to one 30 by 23 by 23 mm., the larger tumors usually showing much necrosis, although not often infected because of the protection afforded by the intact tunica albuginea. Contrary to the experience with other species, no case has been observed in an ectopic testicle, a malformation that seems to be rare in mice. As with other tumors, these growths do not appear until the mice are of middle age or older; the youngest animal in this series was 9 months old when the tumor was first seen and died when 14 months old; the oldest was 3½ years at death, the tumor having been under observation for eight months.

Four tumors seemed to result directly from trauma, appearing in mice while under observation because of injuries to the genital

organs received in fighting. One of these was a typical spindle cell sarcoma (3117) which arose at the site of a bitten wound of the testicle, while simultaneously a similar sarcoma arose in a

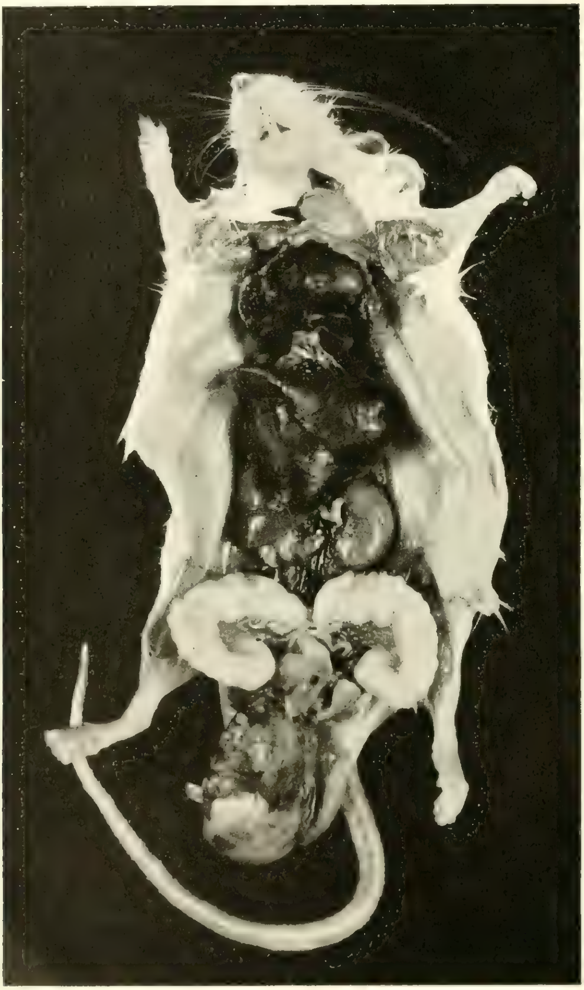

FIG. 2. PRIMARY TUMOR OF THE RIGHT TESTICLE OF A MOUSE, SHOWING THE GROSS APPEARANCE USUAL IN THESE CASES

The lower pole of the tumor is necrotic. It is entirely encapsulated and there are no metastases. Microscopically this was a typical "mesothelioma" or "orchidoblastoma" as shown in figures 3 and 5.

wound on the back. Of the other three, two were of the usual large cell type (3561 and 8145) while one (585) again was a spindle cell growth. It is, of course, impossible to tell how many of the other 24 tumors followed trauma.

Several instances of simple cyst formation, probably secondary to trauma, have not been included in this series.

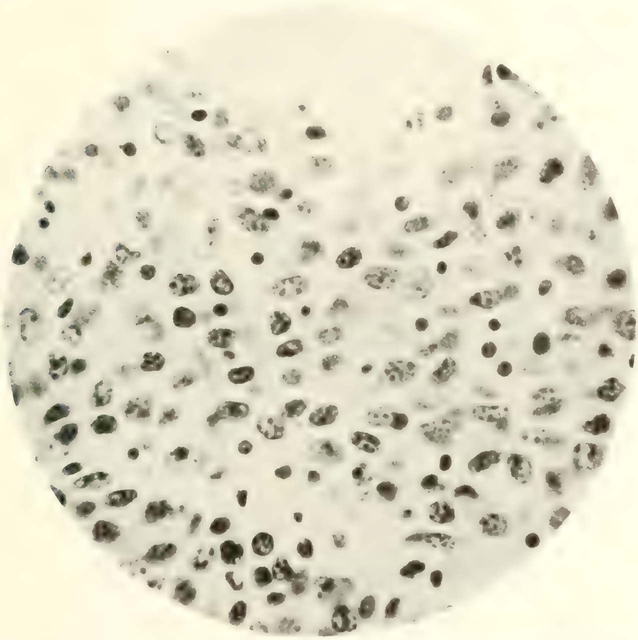

FIG. 3. SHOWING THE TYPICAL CYTOLOGICAL CHARACTERS OF MOST OF THE TUMORS OF THIS SERIES. (× 480)

At the lower edge of the field is a blood-space, showing the direct relation of blood and tumor cells, without distinguishable vessel wall.

Microscopically the great majority of these tumors are all of one structure (fig. 3), which is entirely typical and characteristic. These typical growths are composed of large rather pale and delicately architectured cells. The nuclei are vesicular, often markedly so, with the chromatin in coarse granules and not uniformly arranged. There is an abundant cytoplasm, staining faintly, usually with well defined margins and of polygonal

outline unless compressed into flattened or spindle shaped cells (fig. 4). Mitotic figures are fairly abundant in some specimens, but never extremely numerous; pycnosis and karyorrhexis are frequently seen. Commonly there is a tendency for the cells to be arranged in cords, as in liver tissue, and an alveolar structure is often distinct although never very well developed. As with all mouse tumors, the stroma is seldom so dense as in

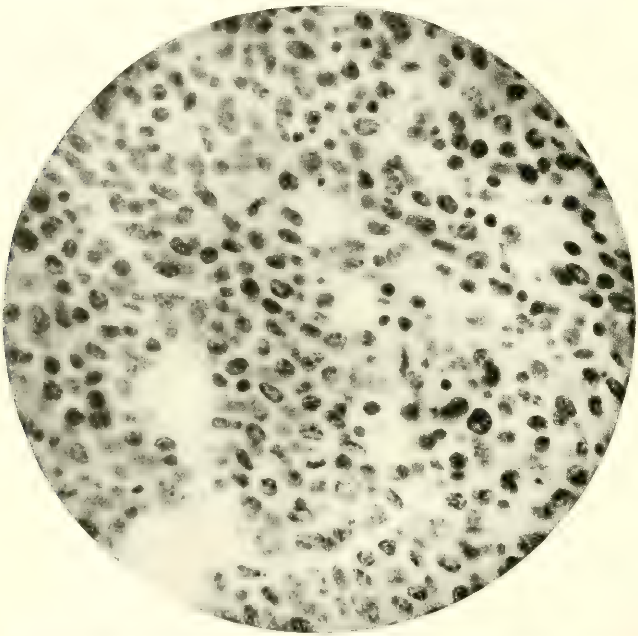

Fig. 4. Tumor Cells Somewhat Compressed Into a More Spindle Cell Type. (× 480)

corresponding human tumors. Usually the tumor is extremely vascular, with large blood channels (fig. 5), the walls of which are often composed solely of tumor cells, although sometimes the vessels have definite fibrous walls; hemorrhages are frequent, and in many sections old pigment and cholesterol masses indicate the site of former hemorrhages. Cells with atypical and giant nuclei are common, and multinucleated cells are often found. Sometimes tubule-like structures are present, but these cannot

be identified as typical "rosettes" such as have been described in human testicular neoplasms. Multipolar and other abnormal forms of mitosis have occasionaly been seen.

Necrosis is present to greater or less degree in most of the specimens, and in some nearly all the tumor is necrotic, as if strangulated by the dense capsule. Often the necrosis is uniformly distributed in those cells more remote from the blood

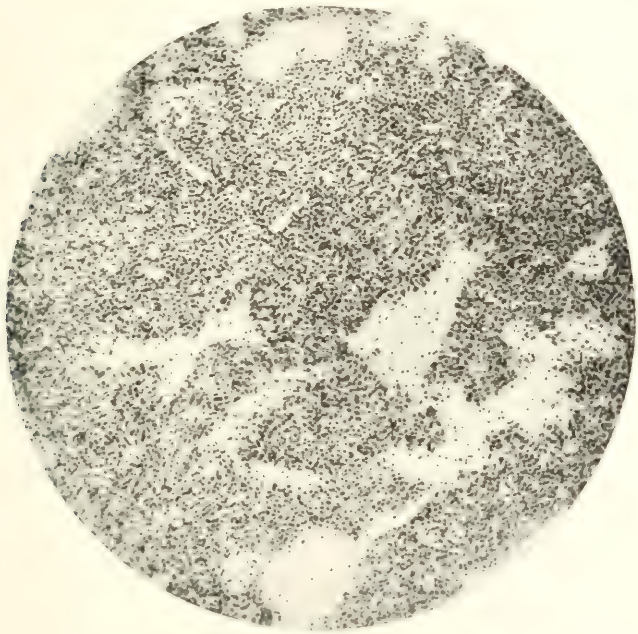

Fig. 5. Showing the General Histological Features Characteristic of Most of the Tumors of the Mouse Testis. (× 110)

The intimate relation of tumor cells to blood-spaces is a striking feature.

spaces, leaving about each vessel a zone of living tumor cells so that the resulting picture resembles a "hemangiosarcoma," often quite characteristically (fig. 6). This recalls the fact that a common diagnosis of testicular tumors in man is "angiosarcoma."

In every case the original testicle tissue has been almost or quite completely destroyed, so that only occasionally can the

remains of a single isolated seminiferous tubule be found. Most often the tubules are only indicated by a partly calcified tubular outline, identified solely by the sperm heads which persist in the lumen. If more tubules remain they are generally compressed against the capsule, often heavily pigmented. The capsule of the testicle always persists, and although usually invaded, it is perforated only in a few instances; occasionally the capsule

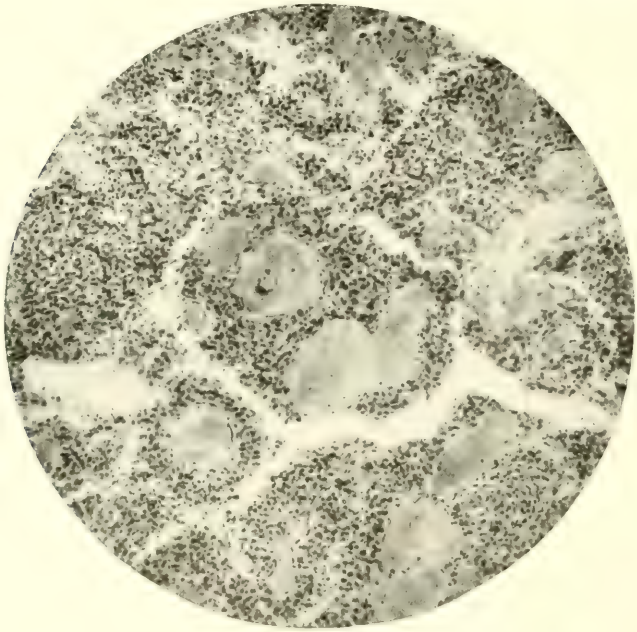

FIG. 6. TESTICULAR TUMOR IN WHICH THE TUMOR CELLS ARE GENERALLY GROUPED ABOUT BLOOD-SPACES, THUS RESEMBLING "ANGIOSARCOMA." (× 110)

contains what seem to be lymph-vessels filled with plugs of tumor cells. When seminiferous tubules remain for comparison, it is usually apparent that the typical tumor cells have much the same structure as some of the cells of the tubule, the spermatogonia.

The stroma is usually scanty and very delicate, so that large areas often show no evidence whatever of stroma elements.

When best defined it divides the tumor cells into indistinct alveoli, and frequently shows an abundance of small lymphoid cells. In many instances clear cyst-like spaces are found without any distinct lining, but giving the impression of resulting from softening and absorption of the tumor, rather than from cyst formation. Blood pigment is often found in phagocytic cells in the stroma and capsule.

Five of the tumors present a histological structure resembling sarcoma, but in three of these, at least, it is probable that the sarcomatous appearance is merely the result of flattening of the cells by pressure. This assumption is supported by the finding, in some of the sarcoma-like tumors, of cell areas resembling the more usual type of testicle tumors, and by the occasional presence, in the ordinary tumors, of areas of spindle cells that are undoubtedly the result of local pressure, e.g., beneath the capsule. These tumors would seem to correspond to the type of tumor often found in the human testicle and commonly diagnosed as alveolar sarcoma or angiosarcoma. One of them (10062), however, presents so much finely divided intracellular golden pigment, without evidence of old hemorrhage as a source of this pigment, that it may possibly represent a tumor derived from the interstitial cells of Leydig, for tumors of such origin have been described in man.

Of the remaining two, one is a typical spindle cell sarcoma which has been described in our paper on sarcoma in mice (case 3117). This mouse was bitten on the back and on the genitals. From each of these sites arose a typical spindle cell sarcoma. The genital tumor seemed to arise from and replace the testicle. Both neoplasms developed at the same time, and seemed to be independent, primary, spontaneous tumors. This mouse also had an adenoma of the liver. The fifth tumor (fig. 7) also seems to be a typical spindle cell sarcoma (7308), in which no persisting elements of testicle can be found; at one point it has grown through the capsule, so that the growth is bilobed. Mallory's connective tissue stain supports the interpretation of the growth as a true sarcoma, the individual cells lying in and evidently producing a matrix of collagenous fibers.

This tumor is especially interesting as being the only growth occurring in an animal not derived from strain 90.

It is a matter for comment that we have found no example of the teratoid type of tumor that is so common in the human testicle. In not a single section have we found a trace of heterologous tissue elements that might suggest a teratomatous origin. Twice we have found simple cysts, apparently resulting

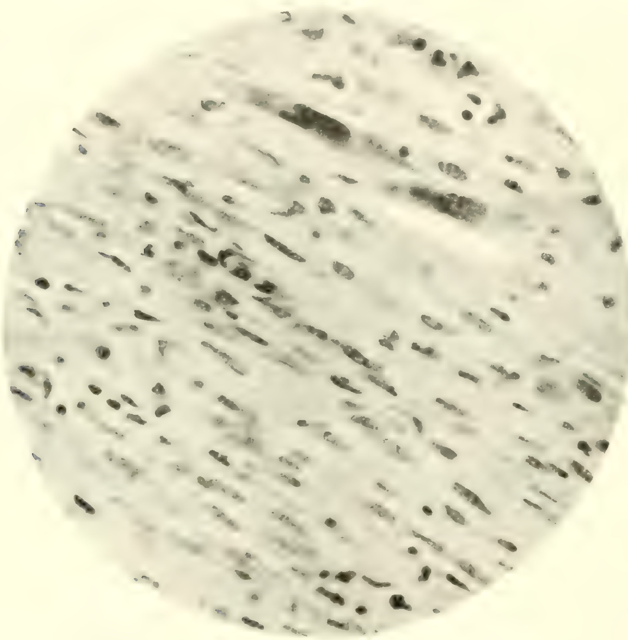

FIG. 7. SPINDLE CELL SARCOMA ARISING WITHIN THE TESTICLE OF A MOUSE. (× 180)

from traumatism and subsequent absorption of the testicle elements. Indeed, in these 19,000 autopsies which have furnished so rich a tumor material, we have found but a single instance of true teratoma, and this arose in the ovary. We have made complete serial sections of three of these testicular tumors, without finding any teratomatous elements. This evidence would seem to us to indicate that, at least in the mouse, the orchidoblastomas do not ordinarily arise in teratomas, which have been

maintained by some to be the common origin of the similar tumors in man. The histological evidence of the similarity of the tumor cells to the spermatogonia would seem to support Frank's view that these neoplasms arise from the epithelium of the seminiferous tubules.

In view of the extremely heterotypical structure of these growths, the total absence of metastases in this series is surprising, especially as metastasis is so extremely extensive and early in the structurally similar tumors of man. And in other mammals, also, metastases are described from testicular tumors, especially in the horse.

One case alone (9441) showed involvement of both testicles, the structure of each growth being the same. In our first canine case, however, the animal showed no recurrence three years after orchidectomy, and the second had developed no visible metastases when autopsied. Several of our sections of mouse tumors show what appear to be plugs of tumor cells in the lymph-channels, while the blood-spaces are commonly lined with naked tumor cells, apparently offering every opportunity for extensive metastasis. Nevertheless, close inspection of the lung sections has failed to show even the retrogressing tumor cell emboli which are so often found in the lung in connection with abdominal carcinoma, as pointed out by M. B. Schmidt.

In but five cases was the tunica albuginea definitely perforated by macroscopic growths. One of these, however, showed very extensive formation of secondary nodules (7870), there being several large tumors outside the testicle (fig. 8). Here the growth consisted of six contiguous nodules, each distinct and enclosed in a capsule. Histologically all these nodules are of similar structure, characterized by an unusually large amount of lipoid material and many necrotic areas. So far as structure is concerned this tumor does not seem to be more malignant than other tumors that do not show the multiple nodules. In one case (2753) the growth extended along the spermatic cord, so that the mass resembled a hernia.

As noted in previous studies of special types of tumors in mice, animals with one tumor are likely to exhibit a growth of

some other sort, apparently more often than would be explained by the law of probability. With testicular tumors the coexistence of other neoplasms would not be expected to be so

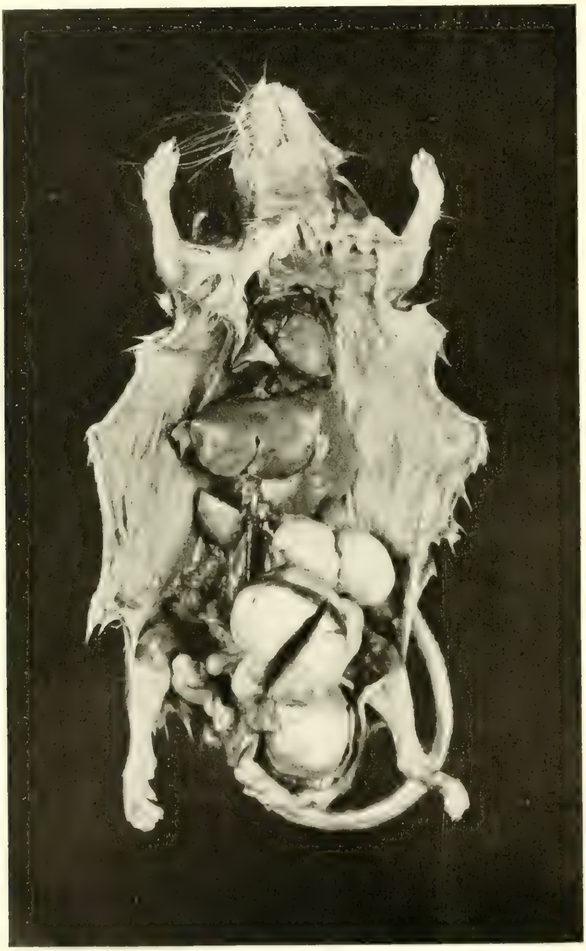

FIG. 8. TUMOR OF THE LEFT TESTICLE WITH THE PRODUCTION OF SEVERAL LOCAL METASTATIC GROWTHS, BUT NO REMOTE METASTASIS

frequent as was observed with the tumors previously described (lung tumors, liver tumors, sarcomas) because the commonest of all tumors in mice, the mammary carcinoma, does not often

occur in males. Nevertheless, among our 28 cases of tumor of the testis we find the following seven instances of multiple tumor formation: One mouse with a subcutaneous spindle cell sarcoma and an adenoma of the liver; three with papillary adenomatous growths arising in the lung; two with osteosarcomas, one arising in the thigh (16370), the other in the subcutaneous tissues near the fore leg (8745). In still another mouse there was a subcutaneous growth in the neck of such a character that a diagnosis has not been made (5037). This growth was about 1 cm. in diameter and 2 to 3 mm. thick, consisting of a mass of cells with strongly basophilic cytoplasm, of a finely granular, "ground glass" character, resembling that of plasma cells. The nuclei, however, are centrally located and have not the characteristic chromatin arrangement of the plasma cell nucleus. The cell boundaries are distinct, and the cells tend to form long rows and strings, recalling the structures sometimes seen in the so-called "lymphangioma hypertrophicum." It is probable that this growth is a true neoplasm, but as long as its nature remains undecided its neoplastic character cannot be definitely determined. The character of the cells is such as to make it improbable that it is a metastatic growth. The occurrence of multiple primary new growths in no less than 25 per cent of these male tumor mice is striking evidence of the existence of a predisposition to tumor formation.

SARCOMA OF THE SEMINAL VESICLE

In the literature on tumors of the lower mammals we have found mention of but one instance of a neoplasm arising in the seminal vesicles. Such growths are also rare in man, Ceelen (23) in 1912 having been able to find reports of but five cases of carcinoma and one of sarcoma, to which he added a case of fibromyoma. We have not been able to find records of any other human or animal cases, except that described by Flexner and Jobling (24) as "originally regarded as a sarcoma; probably a teratoma, from which an adeno-carcinoma was developed." This tumor, which has been used extensively in transplantation,

was found in a white rat, as a tumor "the size of a walnut" attached to the left seminal vesicle, without production of metastases. It was first described as a polymorphous sarcoma, but with included glandular elements. In the course of transplantation the carcinomatous elements developed and replaced the sarcomatous structures, becoming established as an adeno-carcinoma by the twenty-eighth generation.

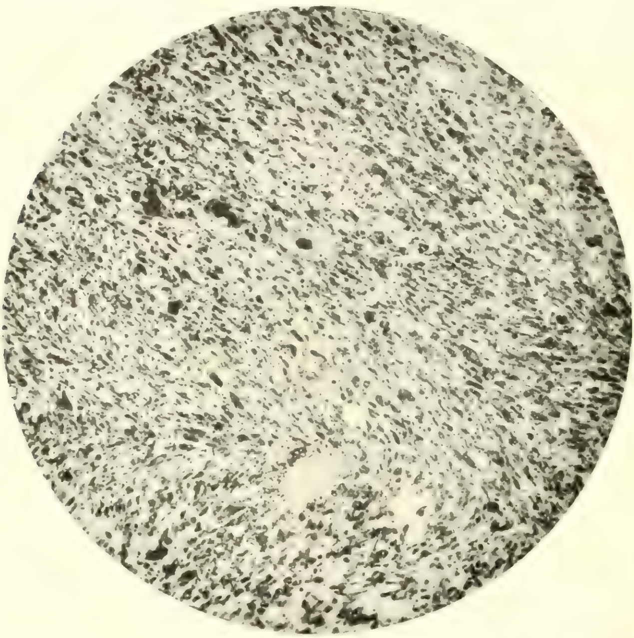

Fig. 9. Primary Sarcoma of the Seminal Vesicle of a Mouse. (× 110)

Note the numerous giant cells, and the polymorphous character of the tumor cells.

A single case of primary tumor of the seminal vesicle has been observed in one of the mice examined in this laboratory (15443). This animal died of pneumonia, and autopsy disclosed the left seminal vesicle constricted at a point midway of its length, with the tip shrunken. The remainder of the organ is distended into a mass 10 by 8 by 8 mm., rather soft, but containing no fluid. The right seminal vesicle is similarly con-

stricted, but there is no tumor tissue and the tip is merely distended with yellow secretion; the rest is in shape like the left, but slightly smaller and yellow in color. There were no metastatic growths to be found. The testicles seem normal, and the kidneys show a slight nephritis. Microscopically the lumen of the right vesicle is found to be distended by a solid cellular growth, limited by the somewhat thickened peritoneal coat and the remains of the muscular coat, except at one point where these structures have been penetrated by the neoplasm. Only a few small groups of columnar epithelial cells remain to represent the original mucosa. The tumor is composed of spindle cells of varying size, but mostly larger than in the usual spindle cell sarcoma (fig. 9). There are also many multinucleated cells, and numerous enormous single cells with a single giant nucleus, sometimes undergoing asymmetric direct division. Thin-walled blood-channels and blood-spaces lined only by unmodified tumor cells are abundant. There are no hemorrhages or necrotic areas, but some cystic spaces full of plasma. This is a typical large polymorphous celled sarcoma. Nothing was found to indicate a teratomatous or carcinomatous character, such as was observed in the Flexner-Jobling rat tumor, yet the fact that theirs was at first diagnosed as a polymorphous cell sarcoma is suggestive. In our case no transplantation experiments were attempted.

SUMMARY

Among 19,000 mice dying natural deaths and examined post mortem, about one-half of which were males, 28 instances of primary tumor of the testicle were found. Most of these resembled in all essential features the tumors that arise in the testicle of man and other animals, consisting of cells closely resembling the epithelium of the seminiferous tubules, arranged in an alveolar structure. Despite great vascularity and a markedly atypical structure, no remote metastasis was observed, although in one case a series of six contiguous independent nodules was formed, and one case showed bilateral testicular tumors. Two of the growths seemed to be true spindle cell sarcomas, one arising at the site of a wound. Three of the typical "or-

chidoblastomas" also followed trauma. No evidence could be obtained that any of these tumors had arisen in a teratomatous growth, and no cases of teratoma have been observed.

One case of polymorphous cell sarcoma of the seminal vesicle of a mouse is described, apparently the second case of a tumor of this organ reported in a lower animal.

Two cases of primary spontaneous tumor of the testicle in dogs are described.

With the exception of one sarcoma, all the 28 neoplasms of the mouse testis occurred in the members of a single strain of mice and its hybrid derivatives, thus substantiating the statement that heredity influences the incidence of tumor development in different organs or tissues. This fact also probably explains the absence of any recorded cases of tumor of the testis in mice from other laboratories.

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THE RELATION OF INBREEDING TO TUMOR PRODUCTION: STUDIES IN THE INCIDENCE AND INHERITABILITY OF SPONTANEOUS TUMORS IN MICE

XIII. PROBLEMS IN THE BEHAVIOR OF TUMORS

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Throughout the range of cancer research, there seems to be no other point so frequently and so completely misunderstood as the subject of inbreeding in its relation to the incidence of tumor. Since the beginning of this series of publications, these researches into the problem of the inheritability of cancer have been met by the statement that "inbreeding increases the number of tumors in a strain," or that "inbreeding is responsible for the high incidence of tumors in a strain of mice, and consequently the demonstration of the inheritability of cancer for mice has no bearing upon the human species, since the latter is not characterized by inbreeding."

The latest and most conspicuous example of this misapprehension of biological procedure and fact appears in Ewing's recent compendium "Neoplastic Diseases," (1) in which he states that "Bashford *attempted* by inbreeding to *intensify* the hereditary influence," and that "Slye *has proven* that *inbreeding* of *tumor-bearing animals greatly increases* the *incidence* of tumors." With this misinterpretation, he dismisses all the exact indisputable experimental evidence for the inheritability of tumors in general and of all types, including cancer, in particular.

It is precisely because inbreeding does not characterize the human species that it is impossible to make any even reasonably complete or accurate study of the inheritability of cancer in

that species, and hence that experimental evidence becomes absolutely necessary as it is impossible to *prove* the inheritability of any character without inbreeding. Mendel in his work with peas was not trying to increase roundness or ovalness, yellowness or greeness, tallness or shortness, or any other quality of peas. He was trying to find out whether these characters were hereditary, and in order to find out whether they were hereditary, he *had to inbreed his peas.*

Cuenot (2) when he crossed the albino mouse with the house mouse, was not trying to increase albinism or greyness or any other quality of mice, he was trying to find out whether pigmentation and lack of pigmentation are hereditary; and in order to do this he *had to inbreed his mice.* He did not thereby *increase* albinism, or *increase* the agouti coat, or *increase* any other character. He demonstrated that pigmentation and albinism are inheritable and in demonstrating that pigmentation and lack of pigmentation are inheritable in mice (whose pigment is melanin) he demonstrated that these characters are inheritable also in guinea pigs, or in rabbits, or in man (in all of which species the pigment is melanin).

Neither Bashford nor Slye has "*attempted to prove*," or "proved that inbreeding increases the incidence of tumors." Neither have Lathrop and Loeb or Little and Tyzzer attempted to demonstrate any such absurdity. All of these experimental workers in cancer have attempted to prove or disprove the inheritability of cancer and they have *all inbred their animals*, because only by inbreeding can you analyze a strain. By inbreeding one discovers what is in a strain, *he does not "increase" or "intensify" anything.*

For the last ten years there have appeared from this laboratory many charts showing the exact genealogical data of strains of mice (families) in which cancer has been transmitted through generation after generation without a break and *in strikingly perfect Mendelian ratios.* At the same time other charts have been published showing the exact genealogical data of families of mice in which there has never been any tumor of any sort, through generation after generation, where the mice have been handled with identical technique *including constant inbreeding.*

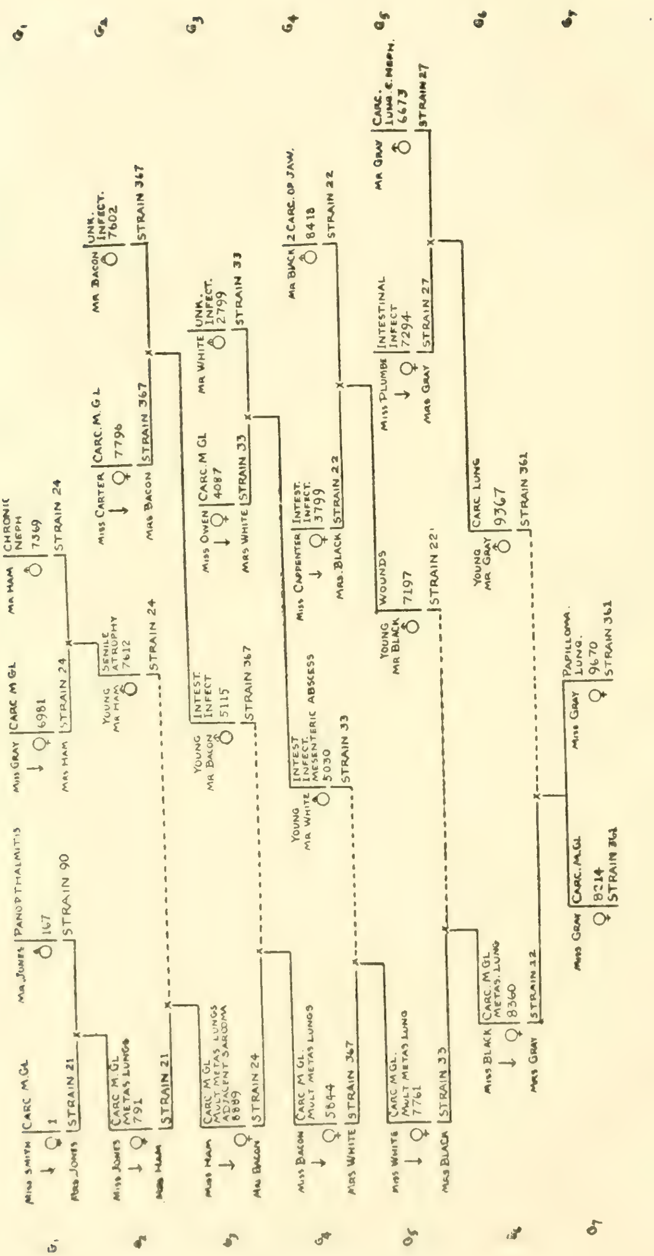
The genealogical charts which have appeared in previous reports from this laboratory have all involved the inbreeding of filial generations of offspring from hybrid crosses, because only so is it possible to find out accurately *just what characters are transmitted in a hybrid cross* and *in just what ratio they are transmitted.* This is the method of all biological procedure in the study of heredity. There is no other method. Just as the analytical chemist does not analyze his unknown by throwing in more unknowns, so the analytical biologist cannot analyze his strain by throwing in more unknowns (by hybridizing) but must inbreed. For the benefit, however, of those who do not comprehend or accept this method, I have drawn up a chart of matings made in this laboratory, which is the duplicate of human mating, and in order that no point in it may escape observation I have decorated it with human nomenclature (Chart 1).

This chart is the exact duplicate of human genealogical charts, in that it contains no inbreeding and not even any consanguineous matings. Miss Smith (female 1) is mated with Mr. Jones (male 167); the offspring of this mating, viz., Miss Jones (female 791) is mated with young Mr. Ham (male 7612) whose medical genealogy is indicated in the chart; etc. Through seven generations one daughter of each pair, is in turn mated with a wholly unrelated male, whose medical genealogy is shown. Every mating in the chart *is a hybrid cross,* just as is the case in all human matings.

Note (1) that carcinoma of the mammary gland entered in the parent female who began this family, viz. Miss Smith (female 1); (2) that the male parent in each generation was the immediate offspring of a cancerous progenitor; (3) that the daughter in every generation exhibited carcinoma of the mammary gland; (4) that lung tumors entered on the male side in the fifth generation; (5) that these lung tumors were exhibited by each succeeding generation (generations 6 and 7).

It must not be inferred from this chart that mammary gland carcinoma can be transmitted only by the female, or lung tumor only by the male. They happened to be so transmitted in this family. But there are many other families in which mammary

CHART I


Miss Smith
Mrs Jones
Carc M Gl
1
Strain 21
Mr Jones
Panopthalmitis
167
Strain 90
Miss Gray
Mrs Ham
Carc M Gl
6981
Strain 24
Mr Ham
Chronic Neph
7369
Strain 24
Miss Jones
Mrs Ham
Carc M Gl Metas Lungs
791
Strain 21
Young Mr Ham
Senile Atrophy
7612
Strain 24
Miss Carter
Mrs Bacon
Carc M Gl
7796
Strain 367
Mr Bacon
Unk. Infect.
7602
Strain 367
Miss Ham
Mr Bacon
Carc M Gl Mult Metas Lungs Adjacent Sarcoma
8889
Strain 24
Young Mr Bacon
Intest. Infect
5115
Strain 367
Miss Owen
Mrs White
Carc M Gl
4087
Strain 33
Mr White
Unk. Infect.
2799
Strain 33
Miss Bacon
Mrs White
Carc M Gl Mult Metas Lungs
5844
Strain 367
Young Mr White
Intest Infect. Mesenteric Abscess
5030
Strain 33
Miss Carpenter
Mrs Black
Intest. Infect.
3799
Strain 22
Mr Black
2 Carc. of Jaw.
8418
Strain 22
Miss White
Mrs Black
Carc M Gl. Mult Metas Lung
7761
Strain 33
Young Mr Black
Wounds
7197
Strain 22
Miss Plumbe
Mrs Gray
Intestinal Infect
7294
Strain 27
Mr Gray
Carc. Lung c. Neph.
6673
Strain 27
Miss Black
Mrs Gray
Carc. M Gl Metas Lung
8360
Strain 12
Young Mr Gray
Carc Lung
9367
Strain 361
Miss Gray
Carc. M Gl
8214
Strain 361
Miss Gray
Papilloma Lung
9670
Strain 361
G1
G2
G3
G4
G5
G6
G7

gland carcinoma was transmitted by the male (note chart 2, strain 146, branch II) and lung carcinoma by the female. Throughout the work of this laboratory no allelomorphism has been demonstrated between sex and type or location of tumor, except, of course, in the sex organ tumors. There is not apparently any allelomorphism even between sex and mammary gland tumors, since this laboratory has yielded a considerable number of males with mammary gland tumors and males who have transmitted mammary gland tumors. The great preponderance of this location of tumor in the female is presumably due to the greater frequency of hyperstimulation of these tissues in the female. Loeb (3) suggests the possibility that the female may be more potent than the male in the transmission of mammary gland cancer. This suggestion is not borne out by the results in this laboratory, wherein the female is no more prepotent in the transmission of mammary gland carcinoma than she is in the transmission of any other character; and the male is just as potent to transmit mammary gland cancer as he is to transmit a grey coat-color. His female offspring demonstrate the inheritance of mammary gland cancer more frequently than his male offspring for the reason stated above, viz., the more frequent chronic irritation of mammary gland tissues in the female. This subject will be discussed more fully in a forthcoming paper.

Innumerable matings of this sort (i.e. like human matings) can be made and their charts published, but it is useless to waste many cancerous mice in such crosses, because they are impossible of accurate analysis. It is best to follow the exact method whereby through analysis one can become acquainted with the intrinsic characters of individual mice and of strains of mice. It is then possible to manipulate the characters of the individual mice or of the strains of mice with a certainty of outcome; entirely eliminating the uncertainty attendant upon the so-called "statistical method," which in reality is not a method at all.

Let us analyze, therefore, some of the charts which have been misunderstood to demonstrate that "inbreeding increases tumor." These charts have been published in reports from this laboratory to which Ewing refers (4), (5), (6). Chart 2 shows the first filial

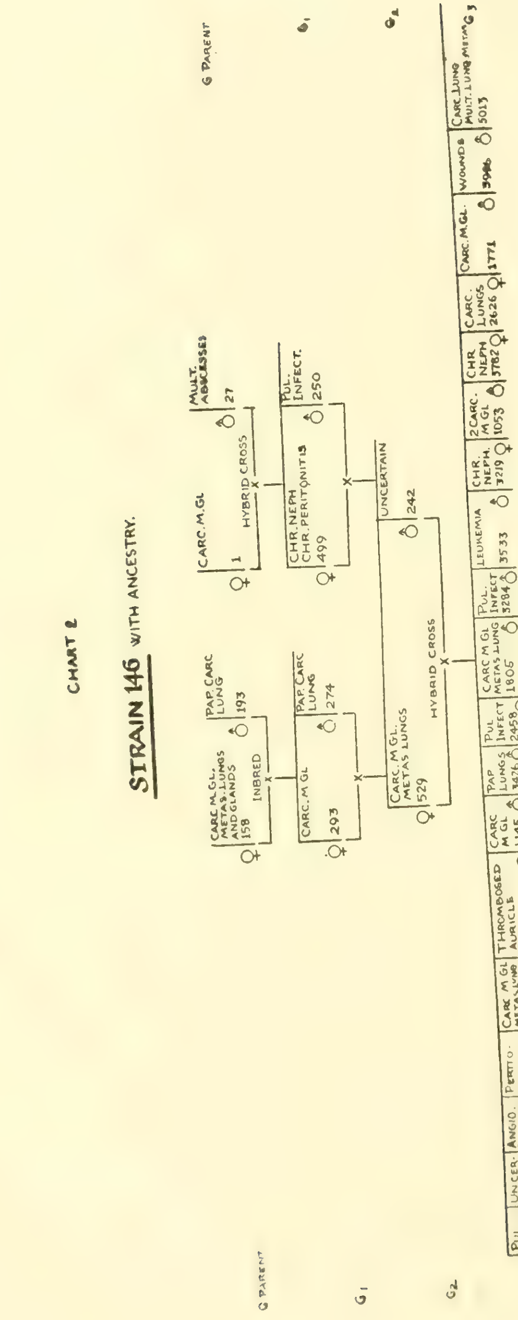


CHART 2
STRAIN 146 WITH ANCESTRY.
G PARENT
G1
G2
G3
CARC. M. GL. METAS. LUNGS AND GLANDS 158
PAP. CARC LUNG 193
INBRED
CARC. M GL 293
PAP. CARC LUNG 274
CARC. M. GL. METAS LUNGS 529
HYBRID CROSS
CARC. M. GL 1
MULT. ABSCESSES 27
CHR. NEPH CHR. PERITONITIS 499
PUL. INFECT. 250
UNCERTAIN 242
PUL. INFECT 2521
UNCERTAIN 2864
ANGIO. SARC M GL 2160
PERITONITIS 1220
CARC M GL METAS LUNG 467
THROMBOSED AURICLE 1236
CARC M GL 1145
PAP LUNGS 3426
PUL INFECT 2458
CARC M GL METAS LUNG 1806
PUL. INFECT 3284
LEUKEMIA 3533
CHR. NEPH. 3219
2 CARC. M GL 1053
CHR NEPH 3782
CARC. LUNGS 2626
CARC. M. GL. 1771
WOUNDS
CARC LUNG MULT. LUNG METAS 5013

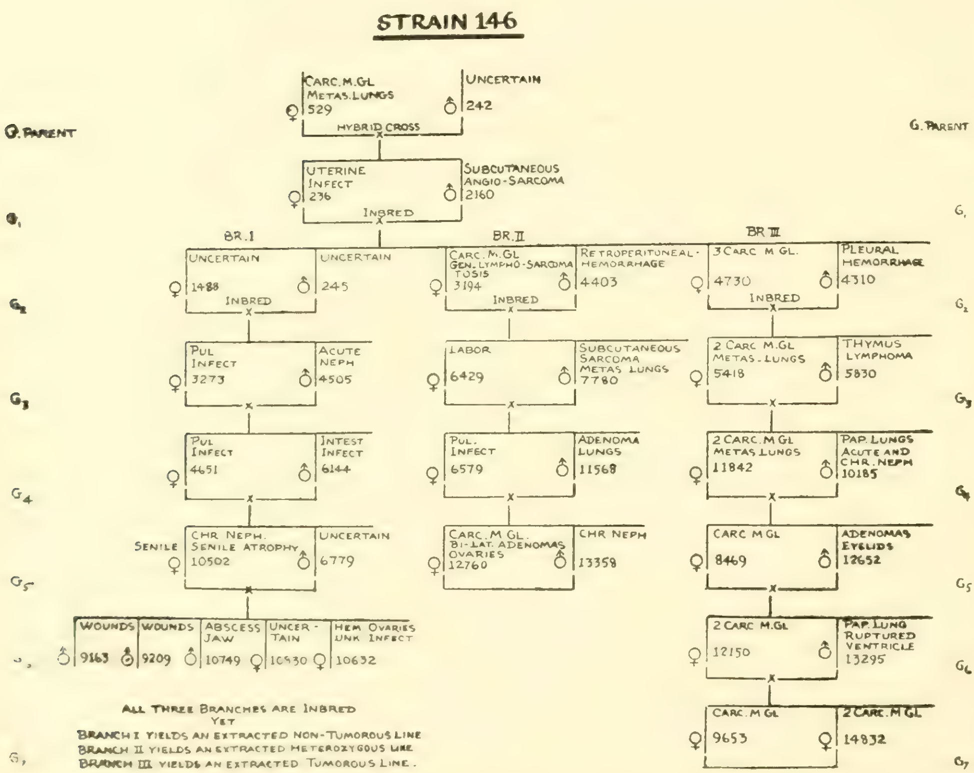
generation of strain 146, with its ancestry for three generations. This strain is the product of a *hybrid cross*, female 529 being in no way related to her mate, male 242. Female 529 died of carcinoma of the mammary gland with metastases in the lungs. Her mother and her grandmother, females 293 and 158, both died of carcinoma of the mammary gland. Her father and her grandfather, males 274 and 193, both died of carcinoma of the lung. She came of a family which carried in all its branches 100 per cent of carcinoma. Her ancestry for two generations was inbred, and we therefore know her to be an extracted cancer-bearing individual, capable of carrying cancer into any strain into which she is mated, with the same certainty with which she will carry albinism. We have then in her, by this method of inbreeding, not an unknown factor, but one whose influence in any compound of which she forms a part can be predicted both in the matter of color and of disease; and whose influence has actually been demonstrated to be just as predicted, both in regard to albinism and in regard to cancer. Her mate, male 242, died of an undetermined disease and had no tumor. His father and his mother, male 250 and female 499, did not exhibit tumor of any sort; his grandmother, female 1, however, died of carcinoma of the mammary gland; his grandfather, male 27, of multiple abscesses without tumor. He comes, then, of a *hybrid*, not an inbred line, with carcinoma of the mammary gland two generations back.

His mating with female 529 is a *hybrid cross* (*not inbred*). Note the number of tumorous individuals in the first *hybrid* generation, viz., nine out of nineteen (of the young who lived to cancer age), the almost exact Mendelian expectation from the mating of an extracted cancerous female with a male heterozygous to cancer (that is carrying it potentially, but not himself developing the disease). A hybrid strain then, which carries 47 per cent of tumor in its first generation (*without inbreeding*) is the product of this cross.

Chart 3 carries on three branches of this hybrid strain 146, and shows what results followed *inbreeding* in each of the above branches.

We have here this same female 529, an extracted cancer-bearing female, mated with male 242 (a heterozygote). The two first-generation hybrids mated in this chart are female 236, a non-cancerous female who died of uterine infection, and male 2160 who died of a subcutaneous angiosarcoma, probably of the

CHART 3



mammary gland. Branch I, *although closely inbred* (brother and sister from the same litter in each generation) never yielded a case of any kind of tumor whatever, *i.e.*, it is an extracted non-tumorous line. Branch II, also closely *inbred in exactly the same way* as branch I, yielded a heterozygous line, the tumors being transmitted sometimes through the male and

sometimes through the female. (Note the mammary gland tumor transmitted through the male in branch II.) While branch III, also *inbred in exactly the same way* as branches I and II, yielded an extracted tumorous line, nearly 60 per cent of these tumors being carcinoma of the mammary gland.

Here are three branches then of the same hybrid family from a cancerous mother, all *inbred in exactly the same way,* yielding three totally different results, *thereby completely eliminating inbreeding as a determining influence* in the incidence or the ratio of tumor production. Moreover, these results are exactly in accordance with Mendelian law.

Ewing (7) states that "the Mendelian characters noted in the heredity of some pathological conditions have not been traced with tumor." All the charts in this paper, and all other charts put out from this laboratory clearly show the "Mendelian character of tumor inheritance." He states further: "the predisposition might be congenital without being hereditary." It is an axiom in genetics that *characters which segregate out,* as do cancer and non-cancer, are hereditary and not congenital. If we do not admit this, no character has ever been proved to be hereditary.

Chart 4 shows strain 164, branch IV, carried out in two families. This is a *hybrid* strain whose progenitors were in no way related. The parent male was a common house-mouse of a strain in my hands many years with no trace of tumor of any sort, although it was *consistently inbred.*

The parent female was a first generation heterozygote of strain 146 (shown in chart 2) who carried cancer into every strain of which she was progenitor, although she herself did not exhibit cancer.

The strain is here shown in two branches *both rigidly inbred.* Branch I is an extracted non-cancerous line, whose members, neither in inbreeding nor in hybridization, ever produced a cancerous offspring. That is, they are proved non-cancerous individuals, whose influence can be predicted in every family into which they are introduced.

CHART 4

STRAIN 164 BRANCH IV

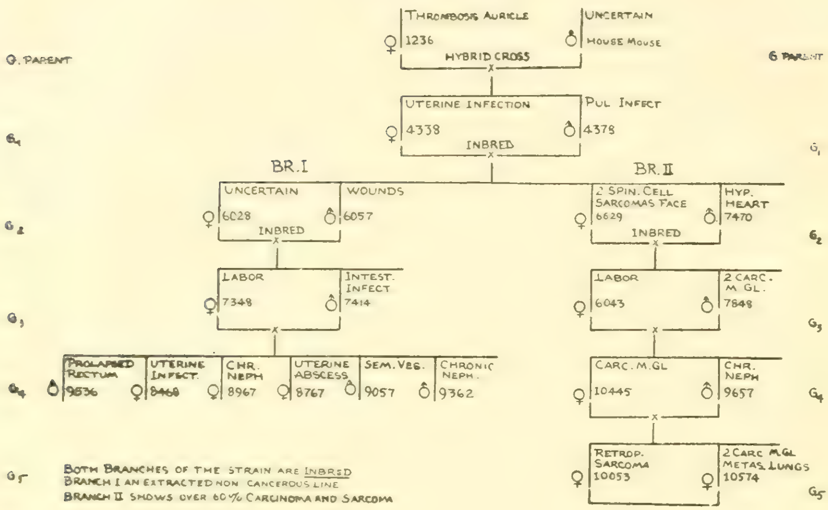
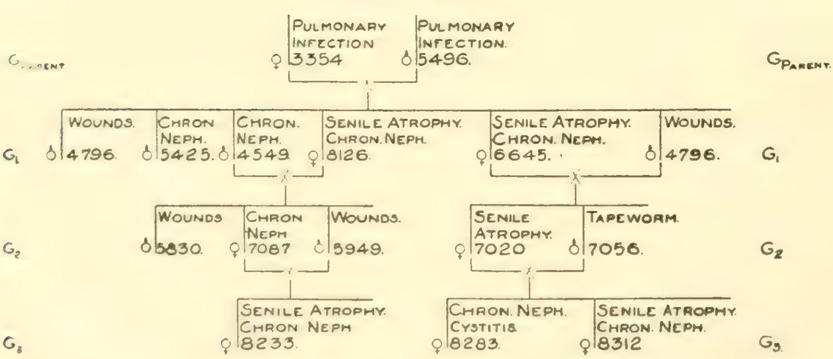


CHART 5

STRAIN 20.



A NON-CANCEROUS STRAIN OF JAPANESE WALTZING MICE
BOTH PARENTS DIED OF PULMONARY INFECTION
THE RESULTING STRAIN SHOWS 0% PULMONARY INFECTION.

Branch II *inbred in exactly the same way* yields over 60 per cent of carcinoma and sarcoma and carries both of these types of neoplasms into every strain into which they are introduced. *Analyzed individuals, then, whose influence can be predicted, are the outcome of the inbreeding method.*

Chart 5 shows strain 20 which has been *closely inbred*, both in the original strain and in its hybrid derivatives, for ten years in my hands, without displaying a tumor of any kind. It is shown in this chart through four generations only; but they are typical of the entire strain and its derivatives, in none of which has cancer or any other tumor ever appeared although the strain has been rigidly inbred.

The foregoing charts are typical. They show conclusively that *inbreeding is not a factor in the increase of tumor*, or in the determination of its incidence; it is merely a method of analyzing a strain in order to determine whether that strain carries cancer or whatever character may be under study.

The real effect of inbreeding upon tumor production seems wholly to have escaped those critics like Ewing who attribute to it any increase in tumor production, although this point was discussed at some length in the Third Report from this laboratory published in March, 1915. It seems advisable, therefore, at this time to demonstrate again as clearly as possible just what is this influence of inbreeding. (Note chart 6, strain 139.)

The parents, female 158 and male 193 (both with carcinoma), were the product of many generations of close inbreeding. They produced only five offspring (compare this with the output of any hybrid cross). These five offspring coming of double cancerous parentage were all cancerous. Four were males and only one a female.

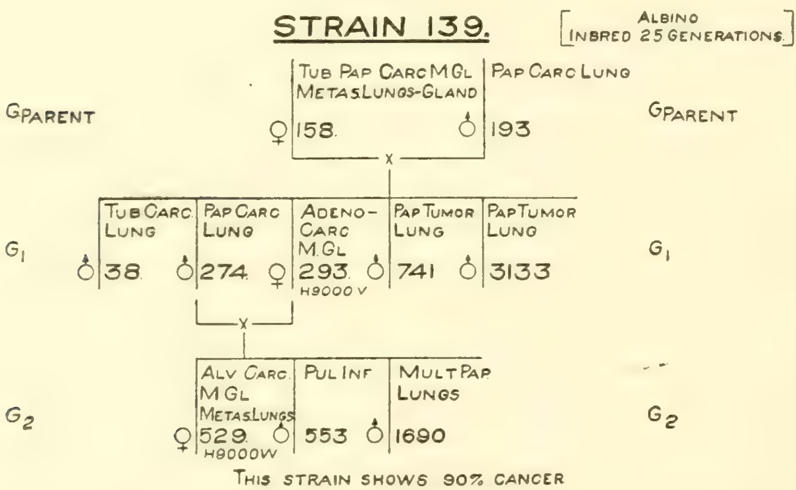
The mating of this one female, number 293, with her brother male 274 (both with carcinoma) produced only three young, two males and one female, all tumorous except the one who died under cancer age, viz., male 553.

The mating of female 529 with her brother male 1690, produced no offspring whatever, and the strain therefore *through close inbreeding was completely eliminated.* Elimination, then, and not increase in tumor, was the result of this inbreeding.

When, however, female 529 who had no young by her brother, was *hybridized* with male 242 (totally unrelated) she became the progenitor of an extremely prolific strain, into which she carried both cancer and albinism. This strain persists in the laboratory to this date, and has in turn furnished the progenitors of many prolific hybrid cancerous strains. This strain, 146, is the one some of whose branches are shown in charts 2 and 3 in this paper.

Again, female 293, first filial generation in strain 139 (chart 6) mated with her brother, male 274 (both cancerous) produced

CHART 6



only three young; mated in turn with her other brothers, male 38 and male 741 (both cancerous) she produced no young. When, however, she was hybridized with male 25 from a totally unrelated strain, she started up a strong prolific line, strain 65, carrying into it both cancer and albinism (shown in two branches in chart 7). Here, again, it was *hybridization which increased the production of tumor; inbreeding which eliminated tumor.*

Instances of this sort could be multiplied in any number desired, but the cases shown in the foregoing charts are entirely typical. *Racially therefore, inbreeding eliminates tumor.*

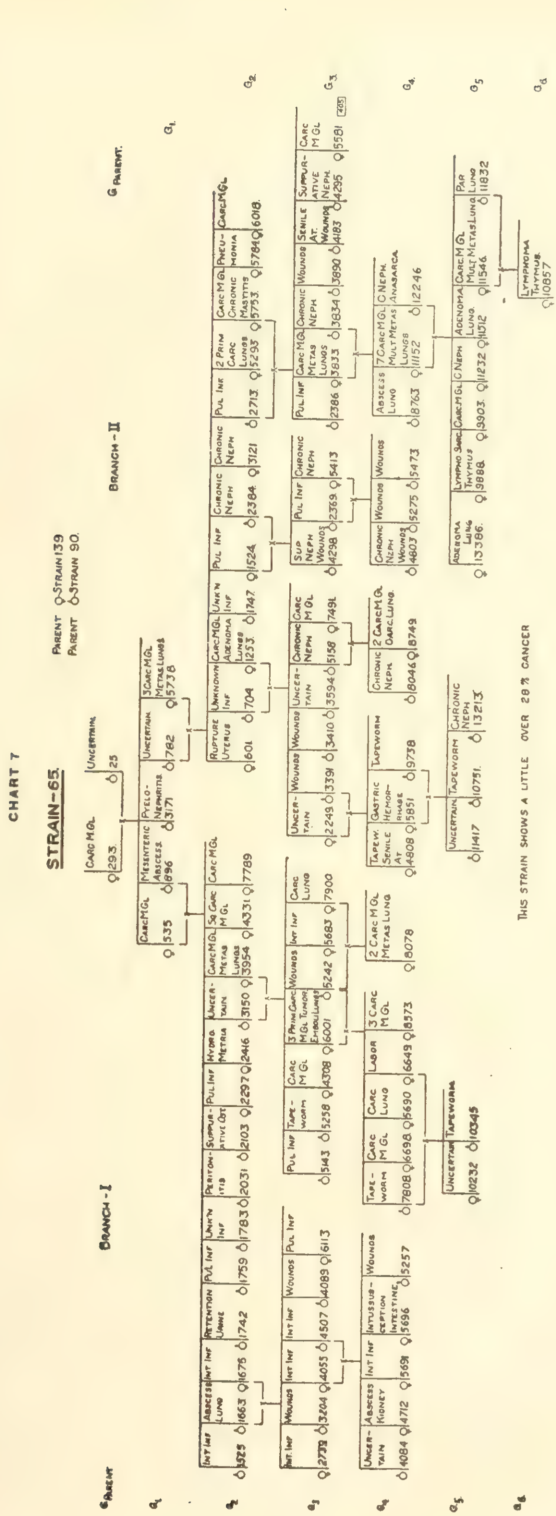


CHART 7
STRAIN-65.
PARENT ♀ STRAIN 139
PARENT ♂ STRAIN 90.
BRANCH - I
BRANCH - II
THIS STRAIN SHOWS A LITTLE OVER 28% CANCER

The literature yields very little on the subject of inbreeding which is of interest here.

Lathrop and Loeb (9) state: "Continued study of strains of mice in which we have established the tumor rate for earlier generations shows that in the majority of cases the rate remains the same throughout later generations. In most of these strains the constancy in the tumor rate is striking. In a few exceptional cases the rate has increased, but in a considerable number of strains there is a distinct fall." This statement, as well as the explanations offered, indicates that the method was, in the main, mass breeding within the stock and not the controlled selective breeding of individuals. The fall or rise of the tumor rate or its static condition within a strain, would thus be attributable to the reasons assigned by the authors or to any one or combination of many other reasons.

Only by the controlled inbreeding of definite individuals, is it possible to eliminate all other influences determining the rise or fall of the tumor rate. Throughout the work of this laboratory the word *strain* has been used to signify a family arising from a mating between two definite known individuals of known ancestry and known cause of death, the subsequent matings within the strain also being between definite, known individuals of known cause of death. Lathrop and Loeb use the word *strain* as this laboratory uses the word *stock*. These authors say further, "As the result of long continued inbreeding, certain characteristics of a strain change. The strain becomes less prolific and less vigorous, and hand in hand with this change goes a lowering of the tumor rate. This occurred in strain 8 and possibly in other strains."

What these authors find to be true of only one strain, I find to be the law in all strains derived as explained above from a single pair, with all later generations derived from the offspring of this one pair, without the introduction anywhere in any generation of any other member even of the same original stock. This method of mating is the only real inbreeding and the only accurate method of testing the effect of inbreeding. Where this method is followed the most complete and exact analysis

possible is made of the characters transmitted by the original pair.

In the Third Report of this series, published in March, 1915, I stated, "Inbreeding if persisted in eventually wipes out any strain which I have handled" (8). This statement remains unqualified to the present date, there never having been an exception to the rule.

The instances cited in this paper are perfectly typical of all of the hundreds of strains handled in this laboratory. This test has been made consistently for twelve years, and from the facts we are justified in drawing this conclusion: *Consistent inbreeding eliminates any strain.*

It has been the pet argument of workers who maintained that inbreeding had no deleterious effect on a strain, that if perfect individuals were selected for the matings no weakness would be transmitted. A pretty theory but wholly opposed to the facts; for if there were these "perfect individuals" some matings of them would have occurred, and in such strains we should have achieved immortality.

The length of time it takes to eliminate a strain by close inbreeding will depend (1) upon whether or not the stocks crossed produce a fertile hybrid stock. For example, the fancy stocks derived in this laboratory from Mus musculus do not produce a prolific hybrid stock when mated with the Japanese Waltzing mouse. Neither does Mus musculus itself produce a prolific stock when mated with the Japanese Waltzer. Many such strains have been produced in this laboratory, no one of which has ever matched in fertility or vigor of progeny the hybrid strains produced by crossing different stocks where both were Mus musculus derivatives. It will depend, also, (2) upon the vigor of the original pair and whether or not they carry any of the same weaknesses or defects; and (3) upon the vigor of each succeeding pair and what same weaknesses and defects they carry, since they are certain to carry some of the same defects.

If either the original pair or any succeeding pair both carry general defects or weaknesses of the respiratory tract, or of the digestive tract or any organ of this tract, or of the circulatory

tract, etc., the strain will quickly run out, since the offspring receive a double dose of this weakness and inevitably transmit it to all offspring. If both original parents or both parents in any succeeding generation carry cancer or any other type of tumor, the strain will be eliminated relatively quickly, since cancer or any other type of tumor interferes with prolific reproduction (10) and since the offspring receive a double dose of cancer and inevitably transmit it.

Inbreeding then, within a cancer strain, speedily eliminates the strain and instead of increasing cancer as some have inferred, eliminates cancer.

This infertility and general inferiority of the strains derived from crossing the Japanese Waltzer with Mus musculus and its derivatives undoubtedly explains in considerable part if not wholly the results of Little and Tyzzer (11) in their studies in the inheritability of tumor "takes" of their implanted carcinoma, J. w. A., in hybrids of Japanese Waltzers and Mus musculus and its derivatives.

The very fact that J. w. A. does not "take" in Mus musculus or its derivatives, indicates a marked difference in these two races. This same marked difference militates also against a vigorous and prolific strain from the hybridization of these two races.

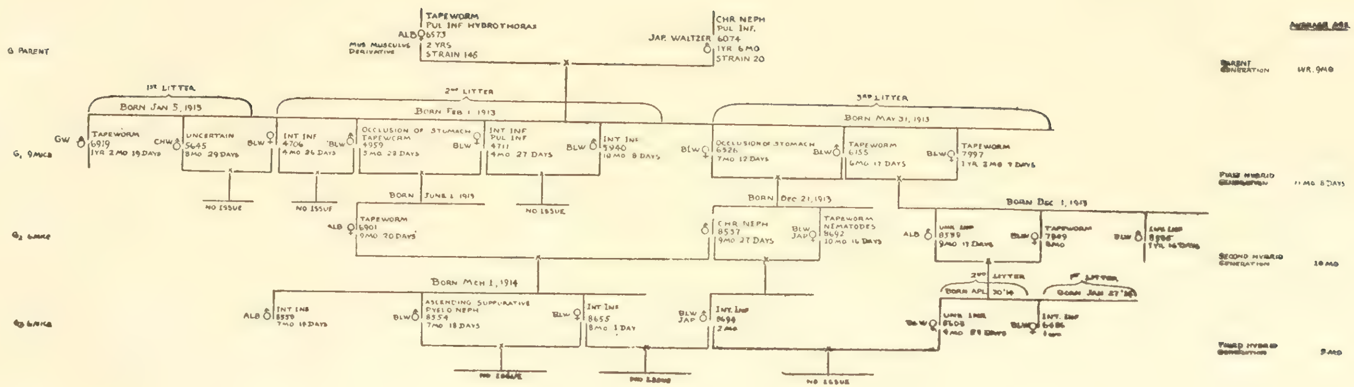
In this laboratory many such crosses have been made. In every case the first generation hybrids have been apparently vigorous mice, inheriting their size from Mus musculus derivatives. But when inbred, they have yielded a very meager output of second generation hybrids, and in no case has it been possible to carry these strains beyond the third hybrid generation, rarely beyond the second. The mice of hybrid generations later than the second are small and apparently entirely infertile when inbred; they are also short lived and relatively inactive.

Chart 8 shows one of these families, strain 479, which is perfectly typical of all the rest.

Strain 479 was produced by mating a Mus musculus derivative, albino female 6573 of strain 145, with Japanese Waltzer male 6074 of strain 20. Female 6573 was a vigorous prolific

CHART 8

STRAIN 479



mouse. Mated within her own strain or hybridized with other Mus musculus derivatives, she yielded prolific sturdy strains.

The parent male 6074, a Japanese Waltzer, mated within his own strain or hybridized with any other Japanese Waltzer strain, produced strains sturdy and prolific as any Japanese Waltzer stock. But these two when cross bred, viz., female 6573 and male 6074, produced a meager strain which died out in the third hybrid generation in spite of every effort to maintain it.

This pair had only 9 young, born in 3 litters; 2 in the first, 4 in the second, 3 in the third. Every possible mating was made of these 9 young, but only 6 second-generation mice were secured. Three of the matings were wholly without issue. In no case was there more than one litter born, and 3 was the largest number of young obtained in a litter, the other two yielding only 1 and 2 mice respectively.

The second generation hybrids in their turn, yielded only 6 third-generation mice. The mating of these third-generation mice gave no offspring whatever and the strain thus died out in the third generation.

The individuals of the first hybrid generation from this cross were slightly smaller than the best Mus musculus derivatives, but larger than Japanese Waltzers. The mice in the second and third generations, however, were considerably smaller than the first generation hybrids and less vigorous by every criterion, viz., scantier coats, feebler pigmentation, less active, less prolific, and shorter lived.

The average age of the parent mice was 1 year 9 months. The average age of the first generation hybrids was 11 months 8 days. The average age of the second hybrid generation was 10 months. The average age of the third generation was only 5 months.

This strain, no. 479, is typical of every strain ever secured in this laboratory in crosses between Mus musculus or its derivatives, and Japanese Waltzers.

The type of mouse derived from these crosses undoubtedly explains the paucity of "takes" which Little and Tyzzer secured

after the first hybrid generation. An examination of their tables shows that in no case did they use any mice beyond the third hybrid generation and the diminution in their number of individuals from the first to the third generation is striking. It is evident that their results in these hybrid crosses were similar to those of this laboratory. It is to be expected that these feeble, short-lived strains would give a constantly diminishing number of "takes" as they give a constantly diminishing number and vigor of offspring. Therefore it is unnecessary to go far afield to find a type of inheritance to fit this number of "takes," particularly where such results conflict with the exact evidence of the inheritance behavior of spontaneous tumors. These authors were dealing with a feeble strain, which neither in offspring nor in tumor "takes" was vigorous like the parent strains. Whatever remains to be explained in their results would be furnished by the fact that in mass breeding the chance selections made for matings might pass by those mice which would have yielded progeny not immune to J. w. A.

To recapitulate then: where the hybrid strain is a feeble one like that resulting from the crossing of the Japanese Waltzer with Mus musculus and its derivatives, inbreeding lowers the progeny and the tumor production with more than normal rapidity, and speedily eliminates the strain.

There remains one point to be discussed in connection with the influence of inbreeding upon tumor production, viz., does inbreeding increase or does it decrease the ratio within a strain between the production of progeny and the incidence of tumor?

Lathrop and Loeb, in their paper quoted from above, state that in general the tumor rate is static within a strain, although in some few strains it shows a rise and in many cases it shows a fall.

Little and Tyzzer, on the contrary maintain that there is a steady decrease in the number of tumor "takes" of increasingly later generations, in their experiments, and deduce therefrom the application of a multiple-factor hypothesis for the inheritability of cancer. Their experiments, as stated above, were limited to three generations.

In considering this conflicting evidence, it must be remembered that Lathrop and Loeb are dealing with spontaneous tumors, while Little and Tyzzer are dealing with grafts. No further evidence of the intrinsic difference between spontaneous and grafted tumors is needed than Tyzzer's own results in later obtaining spontaneous tumors in individuals which had refused grafts (12).

Moreover, as stated above, Little and Tyzzer were obviously not demonstrating the inheritance behavior of "takes" of grafted tumor, but only the biological relation between tumor "takes" and a stock of mice of low grade metabolism and productivity. Their results were exactly what would be expected from such low grade, non-prolific stock.

A very large amount of evidence on the subject of the ratio between the production of tumor and of progeny has accumulated in this laboratory during the many years devoted to the study of spontaneous tumors. Obviously not all of this evidence can be brought within the confines of a single paper.

I have therefore gone over the strains already charted in Reports 5, 7 and 9 published from this laboratory, as these strains were selected with no reference whatever to the point here under discussion, and hence will yield typical and wholly unbiased evidence on the subject.

Chart 9 has been drawn up to show thirty-six of these strains which have already been published giving the number of the strain, the number of generations through which it was charted at the time of publication, the percentage of tumor production in the parent generation and in each succeeding filial generation, and the average rate of tumor production for the entire strain.

For example: Strain 246, charted through 5 generations, showed 50 per cent of tumor in the parent generation, 33 per cent in the first filial generation, 33 per cent in the second filial generation, 66 per cent in the third filial generation and 100 per cent in the fourth filial generation, or an average rate of 56.4 per cent for the entire 5 generations, etc.

Several points must be borne in mind in the study of this chart: (1) The matings made in these strains were all made *to test the Mendelian behavior of cancer;* not to find out how many

CHART 9

RATE OF TUMOR PRODUCTION
IN EACH GENERATION OF VARIOUS STRAINS

	STRAIN	NO. OF GENERATIONS	TUMOR RATE PARENT GEN.	TUMOR RATE F_1	TUMOR RATE F_2	TUMOR RATE F_3	TUMOR RATE F_4	TUMOR RATE F_5	TUMOR RATE F_6	TUMOR RATE F_7	AVERAGE RATE
1	246	5	50%	33%	33%	66%	100%				56 4%
2	245	4	100%	20%	60%	100%					70 %
3	215	5	50%	29%	18%	16%	33%				29 2%
4	65 BR IA	6	50%	50%	50%	50%	0	0			33 33%
5	65 BR IB	5	50%	50%	50%	50%	100%				60 %
6	65 BR IIA	5	50%	50%	50%	50%	50%				50%
7	65 BR IIB	7	50%	50%	50%	50%	50%	100%	100%		64 28%
8	186 BR A	4	50%	100%	100%	100%					87 5%
9	186 BR B	5	50%	0	0	20%	0				14 %
10	202 BR A	5	50%	50%	50%	57%	50%				51 4%
11	202 BR B	6	50%	50%	50%	0	44%	100%			49%
12	112	4	50%	0	50%	50%					37 5%
13	124 BR I	3	50%	29%	25%						34 66%
14	124 BR II	3	0	50%	20%						23 33%
15	196	5	50%	60%	75%	0	100%				57. %
16	201	6	50%	50%	50%	50%	50%	100%			58 33%
17	405 BR B	4	50%	0	75%	50%					43 75%
18	104	5	0	0	50%	33%	100%				36 6%
19	384	3	50%	60%	100%						70 %
20	280	3	100%	100%	100%						100 %
21	139	3	100%	100%	100%						100%
22	281	5	100%	0	38%	50%	100%				57 6%
23	343	3	50%	57%	60%						55 66%
24	164 BR ABC	6	0	0	23%	40%	25%	100%			31 66%
25	450	3	50%	75%	100%						75 %
26	143	5	50%	50%	0	0	20%				24 %
27	413 BR A	5	0	40%	16%	57%	75%				37 6%
28	413 BR B	6	0	40%	20%	20%	16%	0			16 %
29	146 BR IB	8	50%	50%	50%	66%	100%	100%	100%	100%	77 %
30	146 BR IA	6	50%	50%	50%	25%	33%	50%			43 %
31	338 BR I	5	100%	50%	25%	77%	25%				55.4%
32	338 BR III	6	100%	0	38%	36%	75%	100%			58 16%
33	338 BR IX	4	100%	0	80%	50%					57 5%
34	338 BR XI	5	100%	50%	36%	50%	50%				57.2%
35	338 BR XII	5	100%	50%	0	50%	50%				50 %
36	338 BR A XIII	5	100%	50%	0	100%	100%				70 %

tumors could be secured. (2) The matings therefore are of four kinds, double cancerous parentage; single cancerous parentage; parentage cancerous on one side, and heterozygous on the

other; and double heterozygous parentage (the latter tested out to show that individuals may inherit and transmit cancer although they themselves do not exhibit it, just as is the case with albinism). (3) The parent generation, then, in each of these cases shows 100 per cent of cancer, or 50 per cent of cancer, or 0 per cent of cancer, according to what test was being made. (4) As has repeatedly been published from this laboratory, cancer rarely develops in mice under 6 months old, frequently it does not develop until the mouse is 2 or 3 years old; so that the cancer ratio (except in the 100 per cent cases) is nearly always lower actually than it is potentially (*i.e.*, if more mice lived to a greater age more would exhibit cancer). Infections creep in and sweep the mice off in numbers sufficient to lower the cancer rate very considerably.

Examination of chart 9 shows little falling off of the cancer rate except in sporadic cases where infections crept in. On the contrary, the later generations show almost uniformly a tumor percentage higher than those of the earlier generations or than the average percentage for the strain.

Note the percentages shown in chart 10 of the strains under consideration, 83 per cent showed the same or a higher percentage of tumor in generations later than the parent generation, 88 per cent of the strains showed the same or a higher tumor rate later than the first hybrid generation, 93 per cent showed the same or an increased tumor rate later than the second hybrid generation, 79 per cent showed the same or an increased tumor rate later than the third hybrid generation, 88 per cent of the strains showed the same or an increased tumor rate later than the fourth hybrid generation, 100 per cent showed the same or an increased tumor rate later than the fifth hybrid generation, 100 per cent of the strains showed the same or an increased tumor rate after the sixth hybrid generation.

Chart 11 compares each succeeding generation with its immediate predecessor, giving the percentage of strains showing a steadily increasing tumor rate in each succeeding generation. Note the percentages: 58 per cent of the strains showed a higher

tumor rate in the first filial generation than in the parent generation. In 72 per cent of the strains the second filial

CHART 10

PERCENTAGES OF STRAINS SHOWING

RISE OR SAME TUMOR RATE AS PARENT GENERATION	83%
RISE OR SAME TUMOR RATE AFTER F_1	88%
RISE OR SAME TUMOR RATE AFTER F_2	93%
RISE OR SAME TUMOR RATE AFTER F_3	79%
RISE OR SAME TUMOR RATE AFTER F_4	88%
RISE OR SAME TUMOR RATE AFTER F_5	100%
RISE OR SAME TUMOR RATE AFTER F_6	100%

CHART 11

PERCENTAGES OF STRAINS SHOWING

F_1 HIGHER THAN PARENT GENERATION	58%
F_2 HIGHER THAN F_1	72%
F_3 HIGHER THAN F_2	71%
F_4 HIGHER THAN F_3	75%
F_5 HIGHER THAN F_4	88%
F_6 HIGHER THAN F_5	100%

generation showed a higher tumor rate than the first. In 71 per cent, the third hybrid generation exceeded the second in tumor production. In 75 per cent of the strains the fourth

hybrid generation showed a higher tumor rate than the third. In 88 per cent of the strains the fifth hybrid generation showed a higher tumor rate than the fourth. In 100 per cent of the strains the sixth hybrid generation showed a higher tumor rate than the fifth.

We have here then conclusive evidence that as the cancer ancestry behind a generation broadens and deepens, whether by the method of inbreeding or by the method of hybridization, the individuals of that generation tend to run more and more to cancer production. Let us refer again to chart 9 and note how many of these hybrid strains became 100 per cent cancer strains, which originated from 50 per cent cancer parentage (*i.e.*, one cancerous with one non-cancerous parent or one cancerous with one heterozygous parent) or 0 per cent cancerous parentage (*i.e.*, two heterozygotes) strains 246, 65 branch I A, 65 branch II B, 186 branch A, 202 branch B, 196, 201, 104, 384, 164, branches A, B and C, 450, 146 Branch I B.

Note also the cases where 0 per cent cancer strains eventuated from 50 per cent cancer hybrid crosses. These were some of the strains where the purpose was to show that the segregating out of cancer and non-cancer make it possible *speedily to eliminate all cancer from the strain.*

It is obvious that where scientists oppose the theory of the inheritability of cancer and reject the mass of indisputable evidence, it is because they do not wish to accept a theory which they deem unfortunate for the human race; note Ewing's quotation from Le Doux-Le Bard and his personal subscription thereto: "In the interests of the public this doctrine" (i.e., heredity) "ought to be combatted" (13).

If scientists subscribing to this idea would concentrate their attention upon the following facts of heredity, they would find encouragement not hopelessness, in the unquestionable fact of the inheritability of cancer:

(1) In hybrid crosses cancer and non-cancer tendencies *segregate out* and are transmitted as such.

(2) All human matings are hybridizations.

(3) Cancer behaves as a recessive.

(4) It can therefore be wholly eliminated by persistently mating individuals of cancer ancestry with individuals with no cancer in their ancestry.

(5) This elimination is infinitely better than any therapeutic procedure.

(6) The cure of cancerous individuals during the reproductive period, makes it possible for them to transmit cancer to a greater number of progeny (14).

(7) The insistence that cancer is not hereditary, and the continued matings of two cancer-bearing individuals, results in an ever increasing amount of cancer in the human race. If persisted in long enough, such a method will eliminate all the perfectly non-cancerous families.

The progressive increase of cancer in later generations of hybrid crosses demonstrated above, must not be attributed to inbreeding, as exactly the same progressive increase of cancer follows where every mating is a hybridization, if the cancer tendency is bred in constantly in each succeeding generation. In other words, it is *what is put into a mating, not the method of putting it in, that determines which characters shall appear in the offspring.*

CONCLUSIONS

1. Inbreeding is demonstrated not to be an influence in the increase or the incidence of cancer, but merely a method of analyzing a strain.

2. This method of inbreeding is of necessity used by every student of heredity, as there is no test of heredity which does not involve inbreeding.

3. Strains consistently inbred may produce 100 per cent, or 50 per cent or 0 per cent of cancer according to *how much cancer is bred in*, not in accordance with the method used.

4. The real effect of inbreeding is to eliminate cancer by eliminating the strain. It is hybridization which increases cancer by increasing the output of cancer progeny.

5. The ratio of tumor "takes" in increasingly later generations from hybrid crosses of low grade productivity, proves

nothing with reference to the inheritability of cancer, but demonstrates the biological relation between race vigor and the number of tumor "takes."

6. As the cancer ancestry behind a generation broadens and deepens the individuals of that generation tend to run more and more to cancer production. This is equally true of inbred and of hybrid generations, since the amount of cancer which comes out in the progeny depends upon the amount which is put into the ancestry, whether the method is inbreeding or hybridization.

7. It is therefore possible wholly to eliminate cancer from the race by not putting it in through the ancestry; this is true both in inbreeding and in hybridization.

8. In demonstrating the inheritability of cancer and of other tumor types in mice we have demonstrated their inheritability also for man and for every other species in which they occur, since cancer and non-cancer tendencies which *segregate out* in mice must segregate out also in every other species in which they occur, and this is the test of heredity.

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(11) LITTLE, C. C., AND TYZZER, E. E.: Further experimental studies on the inheritance of susceptibility to a transplantable tumor, carcinoma (J. w. A.) of the Japanese waltzing mouse. Jour. Medical Research, 1916, N. S. xxviii, 393.

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Slow Intravenous Injection of Antiserum to Prevent Acute Anaphylactic Shock

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CHICAGO

SLOW INTRAVENOUS INJECTION OF ANTISERUM TO PREVENT ACUTE ANAPHYLACTIC SHOCK *

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From the beginning, the great bane of serum therapy has been serum sickness, the occurrence and severity of which can never be predicted. Individuals who do not give a history of previous sensitization often give reactions varying from death to an uncomfortable urticaria in certain diseases in which repeated doses of serum are given; and on the occurrence of successive infections, each of which call for serum treatment, the serum sickness is much more likely to occur and with greater severity.

Since the recognition of the fact that serum disease is a phenomenon of protein sensitization, or anaphylaxis, much research work has been done to find a means to prevent it, and as a result many suggestions have appeared in the literature, most of which have proved worthless or impracticable. On the basis of the fact that most anaphylactic phenomena are due to the contractions of plain muscle fibers, the administration of drugs acting on the sympathetic nervous system has been advised. Netter[1] advised the use of calcium chlorid, but Rosenau and Anderson,[2] Schippers,[3] and Wentzel[4] showed that it had no effect. Barium chlorid, studied by Biedl and Kraus,[5] and atropin, studied by Auer and Lewis,[6] on sensitized dogs, are uncertain in their action, and at any rate are

* From the Otho S. A. Sprague Memorial Institute and the Department of Pathology, University of Chicago.

1. Netter: Compt. rend. Soc. de biol. **67**: 186, 1909.
2. Rosenau and Anderson: J. M. Res. **21**: 16, 1909.
3. Schippers: Nederl. Tijdschr. v. Geneesk. **2**: 1559, 1910.
4. Wentzel: Centralbl. f. inn. Med. **31**: 697, 1910.
5. Biedl and Kraus: Wien. klin. Wchnschr. **22**: 363, 1909.
6. Auer, J., and Lewis, P. A.: Acute Anaphylactic Death in Guinea-Pigs, J. A. M. A. **53**: 458 (Aug. 7) 1909; J. Exper. Med. **12**: 151, 1910.

not advisable for human use. Rosenau and Anderson show that a narcotic may mask but does not prevent the occurrence of severe or fatal reactions. In studies made for the analysis of the anaphylactic reaction, it was found by Sleeswijk[7] and Friedberger and Hartoch[8] that there was a diminution of complement in the blood of an animal immediately after anaphylactic shock. In order to prove the significance of this diminution of complement, to show whether it is a secondary phenomenon or whether complement takes an active part in the production of conditions that cause death, Friedberger and Hartoch made use of the fact observed by Nolf,[9] Hektoen and Ruediger[10] and others that hypertonic salt solution (from 1.5 to 2 per cent.) will prevent the combination of complement with its sensitized cells. By slowly injecting into sensitized guinea-pigs 0.3 c.c. of concentrated sodium chlorid solution just before the injection of antigen, they were able to diminish anaphylactic shock markedly, saving animals from injections that invariably killed the controls. There are, however, no reports of the use of this method in the prevention of anaphylactic symptoms in man.

Another method worked out for the most part by Besredka[11] depends on the production of the state of antianaphylaxis by giving minute single or repeated doses of antigen followed in three or four hours by the full injection. Besredka shows that the same results can be accomplished by the rectal injection of from 5 to 10 c.c. of serum, in which case there also occurs a slow, gradual absorption of antigen. While no satisfactory method for producing the state of antianaphylaxis in a sensitized person has yet been devised, owing probably to the important quantitative factors shown by the studies of Weil[12] to exist, either of these two methods of Besredka is the one usually employed in cases in which the administration of serum in the ordinary way is believed to be highly dangerous.

7. Sleeswijk: Ztschr. f. Immunitätsforsch. **2**: 133, 1909.
8. Friedberger and Hartoch: Ztschr. f. Immunitätsforsch. **3**: 581, [illegible]
9. Nolf: Ann. de l'Inst. Pasteur **14**: 297, 1900.
10. Hektoen and Ruediger: J. Infect. Dis. **1**: 379, 1904.
11. Besredka: Compt. rend. Soc. de biol. **65**: 478, 1908; **70**: 203, 1911.
12. Weil: J. M. Res. **29**: 233, 1913; **30**: 299, 1914.

Friedberger and Mita[13] have suggested another method, which depends on the very slow administration rather than division of dose. They showed that many times the fatal dose of serum could be given intravenously to sensitized guinea-pigs if the serum was diluted and given slowly by a specially constructed apparatus, over a long period of time.

When it was shown that the danger and intensity of serum sickness was influenced by the size of the dose of the serum, it was an obviously logical procedure to attempt to concentrate the active elements in antiserums into as small a volume as possible. The methods of concentration developed by Gibson,[14] Banzaf[15] and others have succeeded in making it possible to produce antiserums, principally diphtheria antiserum, which have a very much higher concentration of antibodies. However, the antitoxic property of serum is closely associated with the globulins, which are at the same time closely associated with the production of anaphylaxis; and while the process of concentration does enable one to give large doses of antibodies in a small volume and thereby the occurrence and severity of anaphylaxis is lowered, serum sickness still occurs.

In the practice of serum treatment of pneumonia, which is being established more and more as a success, the occurrence of two facts causes the question of serum sickness to loom still greater as an inconvenience. In the first place, the concentration of antipneumonic serum has not been successfully accomplished as it has been for diphtheria antitoxin. And in the second place, the promulgators of the use of antipneumonic serum advise the use of large volumes of the serum (from 50 to 100 c.c.) injected directly into veins. Both of these factors are no doubt responsible for the many reports of serious anaphylaxis reaction, especially from the army camps, where many cases of pneumonia have been treated with antiserum.

A consideration of the possibilities for the prevention of these unfortunate reactions leads one to believe that the method of Friedberger and Mita, the slow

13. Friedberger and Mita: Deutsch. med. Wchnschr. **38**:204, 1912.
14. Gibson: J. Biol. Chem. **1**:161, 1906.
15. Banzaf: Proc. Soc. Exper. Biol. & Med. **7**: 148, 1910.

protracted injections of the diluted serum, is the one best adapted to the conditions. This is especially so since we now have an apparatus in the form of the Woodyatt pump,[16] which is ideally suited for the purpose. This apparatus consists of a syringe operated by an electric motor which can be regulated to deliver accurately as small a volume as 0.1 c.c. of fluid a minute directly into the veins. By varying the rate of injection and the dilution, antiserum may be given as slowly as desired. As a matter of fact, this is one of the first uses that suggested itself for the apparatus; and communications from Woodyatt remark the necessity of the study of this procedure.

Most manufacturers resort to the addition of tricresol or phenol to serums, in order to preserve their products from bacterial contamination. For this purpose, from 0.3 to 0.5 per cent. of phenols are added. This means that the patient may receive with a single injection as much as 0.5 gm. of phenols directly into the blood stream, and with repeated injections, from 1.2 to 2 gm. in the twenty-four hours. Voegtlin[17] finds that the addition of phenols to serum not exceeding 0.5 per cent. does not impart toxic properties; but on the basis of his pharmacologic observations, he concluded that such preserved serums should be injected at very slow rates.

For the experiments, a series of dogs, rabbits and guinea-pigs were sensitized to horse serum; dogs, by being given 10 c.c. subcutaneously; rabbits, by being given 5 c.c. intravenously, and guinea-pigs, by being given 0.01 c.c. intraperitoneally. After the proper incubation period, these animals could be easily killed by a small dose of horse serum given intravenously.

ANIMAL EXPERIMENTS

Dogs.—The most characteristic phenomenon of anaphylaxis in dogs is a primary fall in blood pressure, a reaction that can be easily registered graphically on a kymograph. Sensitized dogs of about the same weight were anesthetized with ether and one-fourth grain of morphin, and kept under anesthesia through a tracheal cannula. A mercury manometer making tracings on a smoked drum was connected to the carotid artery. Injections were made into the saphenous vein. The injection of 5 c.c. of undiluted horse serum all at once into

16. Woodyatt: J. Biol. Chem. **39**: 355, 1917.
17. Voegtlin: Bull. 112, Hyg. Lab., U. S. P. H. S.

the vein caused a marked fall in blood pressure, a slowing of the heart, and slow, deep respirations. Death occurred in about fifteen minutes. Injections were made with the Woodyatt machine of 1 : 10, 1 : 40, and 1 : 100 dilutions of horse serum in 0.9 per cent. sodium chlorid solution. The 1 : 10 dilution at the rate of 0.35 c.c. a minute caused a fall in blood pressure when 0.9 c.c. had been injected. The blood pressure remained low during the five or six hours of injection, but did not kill the animal. The 1 : 40 and 1 : 100 dilutions injected at 0.35 c.c. a minute for five or six hours did not cause a fall in blood pressure during the injections. At the end of these injections when 5 c.c. of undiluted serum were given all at once intravenously, there was a fall in blood pressure showing that the animals were not completely desensitized, but enough so that death was prevented.

Rabbits.—Sensitized rabbits of equal weight were injected with 1 : 10 and 1 : 100 dilutions. The 1 : 10 dilution was injected at about the rate of 0.3 c.c. a minute for about six hours. The temperature at the beginning of the experiment was 40 C.; at the end it was 36 C. This rabbit died within one and one-half hours after the injection. The second rabbit was injected with a 1 : 100 dilution of horse serum. During the first two hours and forty minutes, 44.2 c.c. were injected (about 0.27 c.c. a minute), and in the next three hours, 163.5 c.c. were injected (about 0.94 c.c. a minute). The animal died while the injection was in progress. The third rabbit was injected with the 1 :100 dilution. In eight hours and fifteen minutes, 181.35 c.c. were injected (0.3 c.c. a minute). There were no symptoms of anaphylaxis. The temperature showed no alteration. The animal was well and alive the next day, when 2 c.c. of undiluted horse serum were given all at once intravenously. There were no symptoms and no change in the temperature. When 1.5 c.c. of undiluted serum were given intravenously to a similarly sensitized rabbit that had received no preliminary injection with the machine, a very severe reaction was produced, but not death.

Guinea-Pigs.—Given intravenously, 0.01 c.c. of horse serum injected into two similarly sensitized guinea-pigs caused immediate death. With the machine a 1 : 1,000 dilution of horse serum injected at the rate of 0.3 c.c. a minute into a sensitized animal produced death during the third hour of the injection. Two sensitized guinea-pigs injected intravenously for six hours with a 1 : 10,000 dilution at the rate of 0.35 c.c. a minute (a total of 0.0126 c.c. of serum) showed no acute anaphylactic symptoms, but both animals died during the following day. In one of these animals the temperature rose from 102.8 F. at the beginning of the injection to 105.6 F. at the end of the fourth hour, and fell to 99.4 F. at the end of the injection.

The experiments with the guinea-pigs were not satisfactory because the prolonged intravenous injections into such small animals are very severe operations. The injections, rather than the action of the serum, undoubtedly caused the death of the last two animals.

CONCLUSIONS

Acute anaphylactic shock can be prevented in sensitized experimental animals by giving otherwise fatal doses of diluted antigen intravenously at very slow rates.

So far as these results can be applied to man, it is to be recommended that when immune serum must be given intravenously, it should be given slowly and in a diluted form, the Woodyatt pump serving as an excellent means of doing so. The exact quantitative relations must be worked out experimentally with patients. At present it can only be said that the injections should be made as slowly and the dilutions as high as is convenient or necessary under a given set of conditions.

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THE EFFECT OF FEEDING YEAST ON ANTIBODY PRODUCTION

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This work has been undertaken in the attempt to discover the effect of feeding yeast on antibody formation in the rabbit, with the view to a possible explanation of the therapeutic value claimed for yeast in infectious diseases.

From the time of Hippocrates until the present day, yeast has been used as a therapeutic agent in diseases, particularly in those of an infectious nature. Hippocrates used it in the local treatment of leukorrhea, and later it was used by the uncivilized tribes of France and Germany in the same way. Clinicians in the second half of the last century were interested in both its use and its mode of action, and we find articles in regard to yeast, its dosage and its action in various diseases, appearing intermittently from the time of Mosse[1] in 1852, to the present work of Hawke[2] and his associates. The French clinicians in the last years of the last century and the early years of the present, were particularly interested in its mode of action, and such men as Landau, Beylot, Petit, Nobécourt, and others worked on the problem, but obtained no positive results. Hypotheses in abundance were forthcoming, but the true mode of action was unknown. Debousy[3] postulated a "vaccine" secreted against the microbe of furunculosis. Landau[4] thought there might be a threefold action: a direct cellular antagonism, a mechanical expelling of the organisms causing the disease by the overgrowth of the yeast, and finally the use of substratum for its own benefit to the detriment of the other organisms. Beylot[5] attributed an antiglycemic rôle to yeast. De Backer and Jacquin gave it a phagocytic activity, while Calmette proved that it had no such powers. Petit held that yeast could have no antiseptic power, for all those substances with this power suppress the fermentation of yeast; so that he thought that the most important factor was the vegetable nature of yeast, the fact that it takes up enormous quantities of carbon dioxid and renders the atmosphere toxic for the aerobic bacteria. Vigier[6] considered ferments, vegetable nature, growth, and all other suggestions unimportant, and he believed that some other substance, an alkaloid for instance, was the substance to which the action of yeast in disease was to be attributed. On the other hand, Coirre[7] said that the therapeutic activity was always proportional to the

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[1] Lancet, 1852, 2, p. 113.
[2] Jour. Am. Med. Assn., 1917, 69, p. 1243.
[3] Jour. de méd. et de chir. prat., 1894, 65, p. 476.
[4] Jour. de méd. et de chir. prat., 1896, 67, p. 274.
[5] Thése de Bordeaux, 1896.
[6] Revue de thérap. méd.-chir., 1900, 67, p. 152.
[7] Revue de thérap. méd.-chir., 1900, 67, p. 82.

energy of the ferment, which remained the sole criterium to serve as a guide, since it is unknown whether the therapeutic value resides in the act of fermentation, or whether the presence of the ferment per se, fighting the germs that it meets, has the desired effect. Nobécourt[8] suggested the possibility that other as yet unknown substances might be the true active principles of yeast, but concluded that the vital action of the vegetable was the really important action. Bolognesi[9] did not believe that the zymases themselves were important, but that there was some soluble ferment not precipitated by the alcohol which was the true active portion. Brocq[10] finally concludes that yeast acts as a "modifier of the general state, active enough to render the ground unfavorable for the development of the staphylococcus, but not a specific for it." Lardier[11] has shown that as yet no conclusion has been reached by the French school. Hawke and his co-workers have emphasized the efficacy of yeast in the treatment of furunculosis and other staphylococcus infections. They have given no explanation for its action, but suggest that the laxative value combined with some fixed effect on the intestinal tract may be the cause of improvement that follows its use.

Since all the diseases for which yeast is said to be a specific are induced by staphylococcus, streptococcus, and other pyogenic organisms, and since the recovery from these diseases is due to an increased antibody production, it is logical to suspect that yeast, if it has any beneficial action, has an influence on the yield in antibodies. In order to find if this is so, we studied the effect of the feeding of yeast on the production of antibodies to sheep blood cells in rabbits. We had in mind, also, the possibility of discovery and isolation of a substance which specifically increases the production of antibodies, an accomplishment not yet attained, but the importance of which has been realized in clinical medicine. The experiment has been made three times with a series of new animals each time.

Six full-grown rabbits were used each time, two as controls; four were fed with yeast. The controls were fed as much carrots and oats as they desired. The remaining rabbits were placed in individual cages, and each of them fed daily half a cake of Fleischmann's compressed yeast mixed with ground carrots, and after this had been eaten, as much fresh carrots and oats as the animals would eat. After the first time, this was continued for a period of approximately one month.

In the first series, each animal was injected with 30 c c of fresh whole sheep blood intraperitoneally. This was found to be toxic, so the remaining work was done with washed sheep corpuscles. In the first series, 3 to 4 c c of blood was drawn from an ear vein from each animal every 4 days until the sixteenth day, and the antibodies were titrated after each bleeding. In the table, the figures referring to "Lysin" represent the highest dilution of rabbit serum in question in which complete lysis of sheep corpuscles was produced. The tubes were incubated for 2 hours and then placed in the icebox

[8] Jour. de [illegible] de Paris, [illegible], 12, p. 266.

[9] Revue de thérap. méd.-chir., 1899, 66, p. 695.

[10] Presse méd. 1899, 1, p. 45; Jour. de méd. et de chir. prat., 1900, 71, 896.

[11] Thése de Paris, 1901-1902.

until morning. In each test the total quantity of the mixtures was 0.6 c c, of which 0.2 c c was a 5% suspension of sheep corpuscles and 0.012 c c guinea-pig serum as complement; and the remainder rabbit serum and salt solution. Since the estimation of the lysin was made at various times, and not all at once, the figures must be regarded as only approximately correct with regard to the lysin in the rabbit serum at different bleedings because of the undoubted variations in the activity of the guinea-pig serum used as complement, and in the resistance of the sheep corpuscles employed in the different sets of tests. The results, however, are believed to be accurate enough for the requirements in this case. The figures and curve referring to "Agglutinin" give the highest dilution of rabbit serum in which there was a trace of agglutination, namely a halo around the clumped corpuscles. In the case of "Precipitin" the figure and curve refer to the highest dilution of water laked sheep blood with which the serum of the rabbits formed a definite precipitate after 1 hour at room temperature.

In the remaining experiments, the estimation of lysin and agglutinin was made at the same time so that the concentration of the complement and sheep corpuscles remained the same throughout. The precipitin tests were not made. In the second series of experiments, the animals were bled every 3 days, while in the third series, they were bled every 4 days. The same concentrations were used as in the case of the first group.

From the curves in each case it is seen that in no case is there a higher concentration of hemolysins in the yeast-fed animals than in the controls.

In Table 1 are given the results obtained with the third group of animals which fairly well represent the results obtained with the other groups.

TABLE 1

THE EFFECT OF FEEDING YEAST AFTER THE INJECTION OF SHEEP BLOOD ON THE PRODUCTION OF LYSIN AND AGGLUTININ IN RABBITS

Days after Injection of Antigen	Rabbit 1		Rabbit 4		Rabbit 5—Control		Rabbit 6—Control	
	Lysin	Agglutinin	Lysin	Agglutinin	Lysin	Agglutinin	Lysin	Agglutinin
4	0	192	12	768	6	192	6	192
8	6	1,536	12	1,536	12	3,072	12	1,536
12	12	3,072	12	3.072	24	3,072	24	12,288
16	12	192	96	6.144	96	6,144	96	12,288
20	12	384	96	6.144	96	6,144	96	12,288
24	24	768	48	1,536	96	1,536	96	3,072
28	48	384	48	1,536	48	1,536	48	192
32	0	96	12	192	12	1,537	12	192

The figures under "Lysin" give the highest dilution in which complete lysis of sheep blood was produced. The figures under "Agglutinin" give the highest dilution in which distinct agglutination was produced.

The first series shows that lysin, agglutinin and precipitin formation is greater in control animals than in yeast fed ones over a period of sixteen days. In series 2, lysin formation is considerably greater in the control than in the yeast-fed animals, but the agglutinin formation

is slightly higher in the yeast-fed animals than in the controls. In series 3, the agglutinin formation in both control and yeast-fed rabbits is the same, but in the controls the agglutinin remains in high concentration a longer time in the circulating blood of the control than in the yeast-fed animals. The lysin production is much higher in the control than in the yeast-fed animals.

From our work on the antibodies formed to sheep corpuscles we have been forced to conclude that yeast does not act by increasing antibody formation in the rabbit; in fact, it may even cause a reduction in the amount of antibodies formed as compared with those of the control animals.

Opsonins, the most active antibody infections, were not estimated, but the opsonin curve usually parallels the other antibody curves.

The laxative value of yeast was also found to be lacking, for there was no evidence of it in any of the yeast-fed rabbits of these three series. Their diet remained the same as that of the control animals in every respect save the one additional factor of yeast. We are unable for this reason to agree with Hawke's conclusions in regard to the rôle played by yeast in skin and other diseases; namely, a laxative value combined with a probable fixed effect on the intestinal tract.

The importance of any unknown substances we are not able to discuss. It may be that they are of great importance and play the determining rôle in the favorable effects obtained by yeast. As to alkaloids or other chemical substances, we have felt there is no need or evidence for their consideration.

CONCLUSIONS

The feeding of yeast has no stimulating effect on the production of antibodies to sheep blood in rabbits. The production of antibodies was even less than in controls.

There is no evidence of any effect of yeast on the gastro-intestinal tract of rabbits.

STUDIES ON CIRRHOSIS OF THE LIVER FOLLOWING INTRAPORTAL INJECTION OF TOXIC SUBSTANCES.*

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While there are still many unknown factors which influence the cause and development of Laennec's cirrhosis of the liver, it has been recognized by clinicians that the cirrhosis occurs in connection with the absorption of some toxic substances that act injuriously on the liver cells or the connective tissue or on both. In the literature, we have found numerous experiments recorded in reference to this subject. The investigators, however, when studying this subject usually introduced the toxic substances by such routes as the stomach, subcutaneous tissue, veins (except the portal vein), or by inhalation. There are quite a few authors, nevertheless, who have attempted the direct injection of toxic substances into the portal vein; Afanassijew[1] injected alcohol into the portal vein and found cirrhotic changes in the liver, and Ribbert[2] employed alcohol and ether by the same method with increase of interstitial connective tissue following necrosis of the parenchymatous cells. Recently, Kiyono and Murakami[3] reported the changes in the liver caused by injections of various foreign bodies into the portal vein. Diverse changes observed by them depended upon the degree of irritation causing emigration and proliferation of cells in the walls of the blood vessels and in the interlobular connective tissue spaces.

If we would like to know the primary effect of toxic substances which are considered to be the cause of cirrhosis of the liver it would be better and more accurate to make injection directly into the portal vein. This I did, employing alcohol, an extract of cigar tobacco, an emulsion of killed tubercle bacilli, and an emulsion of killed colon bacilli, for the toxic

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substances. I injected a varying number of doses of these substances into different animals, at intervals of some days. I also injected alcohol and extract of cigar tobacco into the ear vein of other rabbits, for the purpose of comparison.

Injections were made as follows: I first removed the hair from the abdomen, disinfected the surface, and made a regular laparotomy. In all cases the substances injected were warmed to 34° to 37° C.; the first injection was in the portal vein with a Pravatz syringe, but all subsequent injections were made into the mesenteric vein, since this procedure was easier. When I made the first injection I always resected a portion of the liver in order to compare with the final findings, and in some rabbits I resected a portion of the liver just before some of the later injections. Some observers have claimed that spontaneous cirrhosis of the liver is a common condition in rabbits, but there has been found no evidence of spontaneous cirrhosis in any of the portions I resected.

In a few animals the suture in the skin became infected, and sometimes prolonged the time before the next operation, but usually the second dose was repeated in twelve to twenty days. A short time after the last injection I always killed the rabbits; a few, however, died from some unknown cause. Those that died, I examined as soon after death as possible, but could not find any changes which might have produced death.

It may be pointed out that the toxic substances which were injected into the portal or mesenteric veins could not always become equally mixed with the blood by the time they arrived in the liver substance. This might easily explain the variations actually observed in different parts of each liver. As a matter of fact, the cases in which the toxic substances were injected into the ear vein exhibited more uniform changes in all parts of the liver. Incidentally, it might be said that there were no considerable changes in the spleen and that no ascites was recognizable in any animal. Further, it can be stated that, in the majority of cases, the changes are more pronounced in the central and peripheral parts of the hepatic lobules than in the intermediary parts. This might be regarded as due chiefly

to the different conditions of the blood flow in the different parts of the lobules, though sections show no special congestion at the central and peripheral parts except in a few cases.

Alcohol experiments. — In the attempt to produce cirrhosis of the liver, alcohol has been repeatedly employed, because it is believed by clinicians to be the common cause of hepatic cirrhosis. In the experiments with it, different results have been obtained by different investigators. While a few pathologists (Saltykow[4] and Lissauer[5]) have gotten results very similar to Laennec's cirrhosis of the liver with alcohol, there are a good many others (Straus and Blocq[6], and Friedenwald[7]) who have not obtained any satisfactory results at all. Alcohol, when introduced into the body by other ways than intra portal, seldom produced positive results, and it must be said that there is no special constant relation between alcohol and cirrhosis of the liver. The question, then, is not really settl d

For these reasons I employed here a solution of forty per cent alcohol prepared by adding sterilized physiological salt solution to absolute alcohol. In all, nine rabbits were injected, five by way of the portal vein (Table I.) and four by way of the ear vein (Table II.). The ear vein injections were repeated once every two to four days, with gradually increasing doses from four cubic centimeters up to eight cubic centimeters. Each rabbit became intoxicated after each injection, more or less; the intoxication being especially marked in those cases in which the alcohol was injected into the ear vein. We may here state that it became gradually more difficult to inject the alcohol, especially in the second group, on account of thrombosis of the vein, and necrosis which sometimes followed.

I observed various changes in the liver structure, some of which were common to all the rabbits used. In brief, these experiments give the following results.

Two case of the first group show no particular macroscopic changes in the liver, while in the three other cases the livers are somewhat firmer than normal and exhibit marked cicatricial change especially on the edges, together with slight

TABLE I.

Alcohol injections into portal vein.

No. of Rabbit.	Period in Days from Last Injection to the Time Animal Killed.	Number of Injections.	Total Quantity of Alcohol Used.	Total Period of Injections in Days.	Macroscopic Examination.	Microscopic Examination.	
						Interlobular Spaces.	Parenchyma.
1 . . .	14	7	37 cc.	121	Edge of liver is cicatrized and firmer than normal.	Marked proliferation of connective tissue and cell infiltration in the interlobular spaces and central parts of the lobules. A few new bile ducts are observed in the interlobular spaces.	The liver cells are generally large, but their nuclei are variable in size and many of them show fatty and other degenerative changes. The degeneration is especially marked in the peripheral and central portions where we see also many regenerated cells. A moderate number of Kupffer cells show fatty and other degenerative changes, while some of them show a proliferation with irregular nuclei suggestive of mitosis.
2 . . .	18	4	16 cc.	83	Ditto.	Ditto.	Ditto.
3 (died)	1	5	20 cc.	75	Slightly opaque.	Slight increase of connective tissue and cell infiltration. There are few new bile ducts. The inner coat of most of the portal veins is injured.	The cytoplasm of the liver cells has a hyaline appearance, but the nuclei are fairly well stained. Close to the central veins the liver cells exhibit fatty changes.
4 (died)	2	5	22 cc.	87	No marked change.	Ditto.	Cytoplasm of cells appears hyaline, the nuclei incompletely stained with various degenerative changes. Capillaries slightly dilated.
5 . . .	13	3	14 cc.	72	Edge of liver is cicatrized and much firmer than normal.	Marked somewhat irregular increase of connective tissue and cell infiltration. Many new bile ducts. Slight thickening of connective tissue along the walls of the central veins. Many of the large blood vessels in Glisson's capsule are missing, apparently.	The liver cells are generally large, but the nuclei are variable in size. They show fatty and other degenerative changes in the peripheral and central parts of the lobules where we see also some regenerating liver cells. Some Kupffer cells show fatty and other changes. A few exhibit a proliferation with some irregular nuclei suggestive of mitosis.

TABLE II.

Alcohol injections into ear vein.

No. of Rabbit.	Period in Days from Last Injection to the Time Animal Killed.	Number of Injections.	Total Quantity of Alcohol Used.	Total Period of Injections in Days.	Macroscopic Examination.	Microscopic Examination.	
						Interlobular Spaces.	Parenchyma.
1 . . .	4	20	123 cc.	70	No particular changes.	Moderate cell infiltration and slight proliferation of connective tissue. Slight thickening of walls along some of the central veins.	The liver cells at the periphery of the lobules are well stained, while in the other parts they have a slightly pale, dirty appearance. In the central portions they show slight fatty changes.
2 . . .	7	16	92 cc.	62	Ditto.	Moderate cell infiltration and slight increase of connective tissue.	No marked changes except a few fatty changes about the central veins. Many Kupffer cells show fatty changes.
3 . . .	3	16	88 cc.	58	Ditto.	Ditto.	No particular changes except slight fatty changes at the periphery of the lobules.
4 . . .	3	11	65 cc.	43	Ditto.	Slight cell infiltration but no particular increase of connective tissue.	The cells are generally well stained, with slight fatty changes around the central veins. Goodly number of Kupffer cells show fatty changes.

irregular roughening of the surfaces. Considerable shrinkage of the mesentery is observed in most of the cases. In the cases of Group 2, however, we see no particular changes in the liver or any of the other organs macroscopically.

The cases in which I injected alcohol through the ear vein show various changes in the liver, consisting of a greater or less infiltration of cells in every case, these being chiefly lymphocytes, while a few of them resemble fibroblasts; except in one case the connective tissue is also slightly increased in the interlobular spaces, but we can see no new bile ducts. The periphery of the lobules is often infiltrated with connective tissue containing round cells and proliferated Kupffer cells. The parenchymatous cells are generally normal in appearance; a few of them, however, show fatty and other degenerative changes. In all cases the intralobular capillaries are not dilated or congested.

On the other hand, the experiments of Group 1, in which the alcohol would not be very dilute when it arrived in the liver, showed decided changes in the liver, which are much more marked than in those cases where the alcohol was injected by way of the ear vein. In these experiments sections show a considerable increase of interlobular connective tissue, more marked in the region of Glisson's capsule, and a considerable infiltration of round cells together with many new bile ducts. From these interlobular lesions some connective tissue with accompanying cell infiltration extends into the lobules. In the central part of the lobules in two cases a good deal of the parenchymatous tissue has disappeared and been replaced by connective tissue infiltrated with round cells. Thus some of the central veins seem to be obliterated. Extending out from this lesion radially along the hepatic capillaries is some connective tissue with or without infiltrated or proliferated cells, and sometimes in the advanced cases this connective tissue is continuous with that of the interlobular spaces. The infiltrated cells are chiefly lymphocytes, but we find other types of cells in various numerical relations to each other, some of which closely resemble fibroblasts, having a large elliptical clear nucleus. In some cases the blood vessels in

Glisson's capsule show no particular changes, but in others the inner coat especially of the portal veins is injured. The parenchymatous cells show fatty and various other degenerative changes together with regenerative processes as noted in Table I. In every case, except one in which the intrahepatic capillaries are dilated a little, it seems that the capillary walls are rather thick without any dilatation.

In spite of the more frequent injections and the greater quantity of alcohol used in the animals of Group 2 than in those of Group 1, the changes in the liver are, as a rule, far slighter in the former than in the latter. If the alcohol does not change its nature in the circulating blood it may be said that the influence of the alcohol on the liver depends more on its concentration when it arrives in the liver than on the quantity of the alcohol introduced. On the other hand, we must remember that when the alcohol is injected into the portal and mesenteric veins it arrives in the liver only through the portal system, while the alcohol which was injected into the ear vein can reach the liver by both the portal and the hepatic system. Further we cannot tell whether the alcohol has changed its nature in its passage through the portal system or not. At any rate, it is evident that the changes of the liver are due to the injurious and stimulating influence of the alcohol.

If we consider the relation between the cell infiltration and connective tissue proliferation on the one hand and the degeneration or destruction of parenchymatous cells of the liver on the other, we may say that the former usually is the result of the latter, in the interlobular or central lesions, because most of the liver cells remaining in these lesions and a good many of those in close proximity to them exhibit various degenerative changes together with much regeneration. On the other hand, in some lesions the connective tissue and cell infiltration and also proliferation of Kupffer cells has advanced far into the lobule from the interlobular lesions without any particular changes in the liver cells, so that we must recognize a primary appearance of connective tissue proliferation with infiltration of Kupffer cells and round cells without any loss of

TABLE III.

Injections of extract of tobacco into portal vein.

No. of Rabbit.	Period in Days from Last Injection to the Time Animal Killed.	Number of Injections.	Total Quantity of Extract Used.	Total Period of Injections in Days.	Macroscopic Examination.	Microscopic Examination.	
						Interlobular Spaces.	Parenchyma.
1 (died)	1	2	7 cc.	20	Marked post-mortal changes.	No marked changes.	The cytoplasm of the cells appears hyaline. Nuclei almost unstained. Intralobular capillaries slightly dilated.
2 (died)	1	2	10 cc.	27	No particular changes.	No marked changes.	The liver cells stain a pale, dirty color, but their nuclei have normal structure.
3 . .	8	7	32 cc.	117	No particular changes.	Moderate cell infiltration with slight increase of connective tissue. A few new (?) bile ducts are observed.	The liver cells are well stained except that some of them at the periphery of the lobule show a pale color. Marked fatty changes. Close to the interlobular lesions a number of regenerating cells are observed.
4 . . .	12	3	13 cc.	58	No particular changes.	Slight cell infiltration without corresponding increase of connective tissue.	A number of liver cells in the intermediate and central parts are stained a somewhat dirty color.
5 . .	10	7	28 cc.	104	No particular changes.	Ditto.	The cells are stained well except a few, which are somewhat faintly stained, at the periphery of the lobules. Decided fatty changes. Close to the interlobular lesion and adjoining the central veins a number of regenerating cells are observed.
6 . .	14	3	14 cc.	46	No particular changes.	Ditto.	The liver cells are large in size. In the central parts of the lobules they show fatty and other degenerative changes. At the periphery regenerating cells are observed. Some Kupffer cells show fatty changes.
7 . . .	8	4	16 cc.	82	Slight congestion.	Moderate cell infiltration with no increase of connective tissue.	No particular changes except a slight congestion.
8 . . .	12	4	14 cc.	64	No marked changes.	Ditto.	Fatty and other degenerative changes in the central parts of the lobules. At the periphery some regenerating parenchymatous cells are observed. A number of Kupffer cells show slight fatty changes.

TABLE IV.

Injections of extract of tobacco into ear vein.

No. of Rabbit.	Period in Days from Last Injection to the Time Animal Killed.	Number of Injections.	Total Quantity of Extract Used.	Total Period of Injections in Days	Macroscopic Examination.	Microscopic Examination.	
						Interlobular Spaces.	Parenchyma.
1 (died)	1	9	22 cc.	27	Congestion.	No particular changes.	The cytoplasm of the cells appears hyaline, and their nuclei are very poorly stained.
2 . . .	4	22	62 cc.	64	No particular changes.	Moderate cell infiltration with occasionally increase of connective tissue.	In the intermediary and central parts the liver cells stain a somewhat dirty color. A number of Kupffer cells show fatty changes. In the peripheral portions we see some regenerating liver cells.
3 (died)	1	6	19 cc.	18	No particular changes.	Very slight cell infiltration without any increase of connective tissue.	The cytoplasm of liver cells appears hyaline. All nuclei are very pale.
4 . .	1	15	48 cc.	53	No particular changes.	Moderate cell infiltration with no increase of connective tissue.	In the peripheral portions some regenerating cells are observed. In the intermediate and central parts the cells are stained a slightly dirty color. Some liver cells and a number of Kupffer cells in the intermediary portions show fatty change.
5 (died)	3	12	35 cc.	30	Slight congestion.	Very slight cell infiltration with no increase of connective tissue.	The cells are stained a pale color. Slight congestion.

the parenchyma. In a word, there may be two kinds of processes at the same time with a difference of degree, — a primary necrosis of parenchyma with secondary connective tissue proliferation and cell infiltration, and a primary connective tissue proliferation with very little degeneration and regeneration of the parenchyma.

Now there will be no time for regeneration of the tissues if alcohol comes too often to the liver through the portal vein in a concentrated condition, but in my experiments there was some interval of time between the injections, and hence the degenerated or destroyed parenchyma could be regenerated and replaced by connective tissue to some extent. Thus after many injections there will be at last a decided increase of connective tissue in the places where the parenchyma has been destroyed as a result of the toxic influence of the alcohol together with its stimulating effects. In point of fact, however, the extent of these cirrhotic changes is not always proportional to the frequency of the injections in the same rabbit or in different rabbits. This may be explained by the observation that the cells once injured by some toxic substance or regenerated after destruction may have an increased resistance to the same toxic substances, though in the different rabbits it depends more or less on the individuality of the animal.

Among others who have experimented with alcoholic cirrhosis may be mentioned Afanassijew, who injected alcohol into the portal vein in one large dose and obtained a liver having the same structure as " Kleinherdige Lebercirrhose." With injections of alcohol and ether into the portal vein, Ribbert obtained an increase of connective tissue and new bile duct formation following necrosis of the parenchyma. Even if my results are not closely analogous with human cirrhosis the experiments show decided cirrhotic changes in the liver, especially marked in three cases.

Nicotine experiments. — Hayami[8] introduced an aqueous extract of tobacco into the stomach of rabbits, guinea-pigs and rats to see what effect it would have on the liver, with the idea that the walls of blood vessels and different organs are harmed

by smoking. In the rabbits he observed very often a congestion in the hepatic lobules and an infiltration of round cells in the interlobular spaces, together with some degenerative changes in the liver cells, chiefly about the central parts of the lobules. He stated that he saw only in two cases among hundreds a marked increase of connective tissue in the interlobular spaces, and he believed that at least one of these occurred as a consequence of the toxic influence of nicotine.

In my experiments the extract of cigar tobacco was prepared by adding fifty cubic centimeters of physiological salt solution to one gram of a certain brand of cigar tobacco, and after leaving it twenty-four hours at room temperature it was filtered through a Berkefeld filter. In eight rabbits the filtrate was injected by way of the portal vein (Table III.) while in five others it was injected by way of the ear vein (Table IV.). The ear injections were repeated once every two to four days, with gradually increasing doses from three to five cubic centimeters. The rabbits developed marked reactions, convulsive in nature, very soon after each injection; the reactions being most pronounced in those that were injected by way of the ear vein.

Macroscopic examination shows no particular changes in the liver or any of the other organs in the cases of Groups 1 and 2, except slight congestion in one case of Group 1 and in two cases of Group 2. In one case of Group 1 there were marked post-mortal changes.

Now, even in the first group of my nicotine experiments, in which the extract of cigar tobacco could not have been very dilute when it arrived in the liver, there was usually just a slight infiltration of round cells in the interlobular spaces with a slight increase of connective tissue in one case, a little increase in thickness of the walls about the central veins in a few others. From the interlobular lesion one can see often proliferated or infiltrated cells extending into the lobules. The branches of the portal veins in Glisson's capsule show sometimes slight inflammatory changes, while no particular changes in the hepatic ducts are seen; but in the interlobular spaces of one case there are a few bile ducts which seem to be newly formed. The changes in the interlobular spaces and along the

TABLE V.

Injections of sterilized emulsion of tubercle bacilli into portal vein.

No. of Rabbit.	Period in Days from Last Injection to the Time Animal Killed.	Number of Injections.	Total Quantity of Emulsion Used.	Total Period of Injections in Days.	Macroscopic Examination.	Microscopic Examination.	
						Interlobular Spaces.	Parenchyma.
1 (died)	3	1	3 cc.	4	No particular changes.	Slight cell infiltration with no increase of connective tissue.	The cells are stained like hyaline, while the nuclei are quite pale.
2	11	5	22 cc.	63	No particular changes.	Marked infiltration and proliferation of cells and increase of connective tissue. Sometimes giant cells are observed in these lesions. Slight increase of connective tissue along the walls of the central veins.	Fatty changes at the periphery of the lobules. Slight congestion. A number of tuberculous lesions are observed in the lobules.
3	14	3	13 cc.	51	Slightly firmer and smaller than normal.	Ditto.	Liver cells are well stained. Some tuberculous lesions are observed.
4	8	2	6 cc.	23	No particular changes.	Ditto.	In the central portions of the lobules some of the nuclei of the liver cells stain a pale and a dirty color. Slight fatty changes at the central parts. Tuberculous lesions are observed. Slight cell infiltration is seen about some of the central veins.
5 (died)	9	2	8 cc.	34	Congestion. Slightly firmer and smaller than normal.	Ditto.	Marked congestion. In the central portions some nuclei take a pale or dirty color. Slight fatty changes about the central veins. A number of tuberculous lesions are observed. Slight cell infiltration about some central veins. Nuclei of the liver cells at the periphery of the lobules are usually quite large, and we see often regenerating cells.

central veins occur evidently as a result, chiefly, of the irritation and injurious influence produced by the extract of cigar tobacco. The toxic effects upon the parenchyma of the liver produced by the extract of cigar tobacco seem not so severe as in the case of alcohol, for the degenerative and also regenerative changes are usually slight; however, the two cases in which the extract was injected many times over a long period of time show considerable fatty changes. The cases in which we see some changes in the parenchyma, show them, however, more marked, especially in the peripheral and central portions of the lobule, where probably the extract acted for a longer period of time or else arrived in greater concentration, though no congestion was observed in any case except one. The extent of the changes produced also is not always proportional to the frequency of the injections in the same rabbit or in different rabbits. This would be explained in the same way as it was explained in the alcohol experiments.

The results in the second group were somewhat analogous to those of the first group, though not quite so severe. The variation in the results obtained between the intraportal injections and the injections into the ear vein of the extract of cigar tobacco may be explained, again, as was explained in the alcohol experiments.

Thus we can produce evidently a toxic effect on the liver by the repeated injection of extract of cigar tobacco into the portal and mesenteric veins, but the results are quite different from those of Laennec's cirrhosis of the liver. We may say, therefore, that there is no apparent relation between cirrhosis of the liver and nicotine.

Tubercle bacilli experiments. — It is believed, chiefly by French authors, that tuberculosis may have a causative relation to hepatic cirrhosis. Many investigators (for instance, Hanot and Gibbert,[9] Storek,[10] Catsaras[11]) described hepatic cirrhosis of animals in tuberculosis, caused by subcutaneous or intraperitoneal injection of tubercle bacilli. Evans, Bowman and Winternitz[12] and recently Kiyono and Murakami[13] injected tubercle bacilli into the portal vein to study the histogenesis of

the tubercle in the liver, and obtained some illuminating results.

The following is a description of my own experiments with tubercle bacilli.

An emulsion of tubercle bacilli was prepared by adding sixty cubic centimeters of sterile physiological salt solution to one-tenth gram of human tubercle bacilli, which had been cultivated two weeks on a five per cent glycerol agar, heated twice for forty minutes at 70° C., and then tested for sterility. I injected this emulsion into five rabbits by way of the portal vein. There was no particular reaction following the injections, although some rabbits died within a few days.

Macroscopically, the livers of this group of experiments show no particular change except that in two cases they are slightly firmer and smaller than normal and that in one there is some congestion.

In these experiments we see marked changes in the liver, namely, infiltration and proliferation of various cells in the interlobular spaces. There are also a number of almost the same kind of lesions within the lobules with or without direct connection with those in the interlobular spaces. The cells in the lesions are chiefly lymphocytes and epithelioid cells in various numerical relations, and we find also sometimes giant cells, which resemble very closely those of the tubercle, but we see no caseation in any of the lesions. The connective tissue observed is best developed at the peripheral edges of the interlobular lesions. From the lesion in the lobules one can see an infiltration of round cells accompanied usually by connective tissue along the intralobular capillaries. We often see, moreover, cells which have infiltrated or proliferated along these intralobular capillaries without, however, the presence of any connective tissue. There are a few giant cells in the lobules sometimes close to the lesions or again at some distance from them. These giant cells may have some relation to the Kupffer cells in their origin. Along the walls of the central veins, except in one case, we notice a more or less increased connective tissue. The blood vessels in Glisson's capsule show usually no particular changes except that in one case they are

completely filled with red blood corpuscles. No new bile duct formations are seen in any case. There are also some toxic effects upon the parenchyma of the liver, that is, there are slight fatty and other changes in the parenchymatous cells, while a number of Kupffer cells are large and multi-nucleated, and a few liver cells are in the process of regeneration.

It is quite clear that the inter- and intra-lobular tuberculous lesions may have occurred where the emulsion of killed tubercle bacilli remained for a longer period of time or where it came in large quantities. The various cells, namely lymphocytes and epithelioid cells, accumulated and proliferated as a result of the injurious and irritating influence produced by the killed bacilli and their toxins. Thus we may produce tuberculous lesions in the liver with intraportal injections of killed tubercle bacilli without the presence of any living tubercle bacilli. Further, though these lesions will be reduced to some extent by reparative processes, such injections will be followed by an increase of connective tissue. In fact, we recognize some tuberculous cirrhotic changes; the older lesions exhibiting much proliferation of connective tissue in the liver, even though it is not proportional to the frequency of the injections. This may be again explained in the same way as it was explained in the alcohol experiments. Therefore, as it was pointed out chiefly by French authors, it is believed that there may be some relation between tuberculosis, expecially of the peritoneum and intestine, and cirrhosis of the liver.

Colon bacilli experiments. — An emulsion of colon bacilli was prepared by adding ten cubic centimeters of physiological salt solution to a culture of colon bacilli which was cultivated on five per cent glycerol agar for twenty-four hours, heated twice at 65° C. for forty minutes, and tested for sterility. There were no particular reactions immediately after the injection, but some of the rabbits died within a few days.

Necropsy shows the liver of one case slightly firmer than normal, with some congestion; but in the other cases there are no particular macroscopic changes recognizable.

TABLE VI.

Injections of sterilized emulsion of colon bacilli into portal vein.

No. of Rabbit.	Period in Days from Last Injection to the Time Animal Killed.	Number of Injections.	Total Quantity of Emulsion Used.	Total Period of Injections in Days.	Macroscopic Examination.	Microscopic Examination.	
						Interlobular Spaces.	Parenchyma.
1 (died)	1	4	16 cc.	61	Slight congestion.	Marked infiltration of round cells with slight increase of connective tissue. Slight thickening of connective tissue along the central veins.	In the intermediate and central parts most of the cells are stained a very pale color. A few liver and Kupffer cells in the central portions exhibit fatty changes.
2	11	3	9 cc.	13	Congestion. Slightly firmer than normal.	Marked cell infiltration with slight increase of connective tissue and a few new (?) bile ducts.	Marked congestion. At the periphery some regenerating cells are observed. Near them we see a number of large vacuolated cells with various degenerated nuclei. In the intermediate and central portions there are degenerated smaller cells. These two kinds of degenerated cells show marked fatty changes.
3 (died)	2	1	3 cc.	2	No particular changes.		The liver cells have become hyaline and their nuclei are very poorly stained.
4 (died)	9	2	6 cc.	28	No particular changes.	Marked infiltration of round cells and slight increase of connective tissue.	A few liver cells are stained pale in the intermediary portions, where slight fatty changes are observed.
5 (died)	A few minutes.	1	4 cc.	A few minutes.	No particular changes.	No particular changes.	No particular changes.

In the interlobular spaces of this group of experiments, except in two cases, we observe a wide infiltration of round cells most marked about Glisson's capsule, accompanied usually with a more or less increased connective tissue. The infiltrated cells are chiefly again lymphocytes, but these are mixed with other cells, most of which have large, clear, elliptical nuclei. The branches of the portal vein in Glisson's capsule show sometimes a slight inflammatory change and in one case we observed in the interlobular spaces a few bile ducts which may be newly formed. In the parenchyma of the liver in these experiments we see also fairly marked degenerative changes following an injection of an emulsion of killed colon bacilli into the portal and mesenteric veins, as indicated in the table. In all these cases except two the Kupffer cells are stained normally, but many of them are large and often multinucleated. Furthermore, some of them are rounded and are often broken loose from their normal positions.

The infiltration of round cells and the proliferation of connective tissue in the interlobular spaces, and furthermore all the other changes in the parenchyma, may be explained almost in the same way as they were explained in the experiments in which tubercle bacilli were employed. At any rate we could see here, in these experiments, also, some cirrhotic change in the liver following the injections of an emulsion of killed colon bacilli into the portal and mesenteric veins.

There have been authors who attempted to produce cirrhosis by an introduction of bacteria by various routes, except the portal vein, and have obtained often positive results. Opie[14] introduced chloroform into the stomach at the same time that he injected colon bacilli into the jugular vein, and observed cirrhotic changes in the liver with new bile duct formations.

When we consider these experiments of other authors with our own, we come to the conclusion that killed colon bacilli and their toxins may produce a cirrhotic change in the liver.

SUMMARY.

1. In the cases in which I injected alcohol into the portal and mesenteric veins repeatedly there were seen marked cirrhotic changes in the interlobular spaces, with often newly formed bile ducts, especially marked in one case; and in addition in two of the cases there was also a marked round cell infiltration with increase of connective tissue in the central portions of the lobules. Further, we saw also in this series various pronounced degenerative changes of the liver parenchyma. The cirrhosis, however, was not the same as Laennec's cirrhosis of the liver. On the other hand, in the series of cases in which we injected alcohol repeatedly into the ear vein, we observed but slight changes in the interlobular spaces and in the parenchyma of the liver. The difference observed in the two groups of experiments may be due to the difference in concentration of the alcohol as it arrives in the liver.

2. It is evident that the repeated injections of extract of cigar tobacco into the portal and mesenteric veins may have some toxic effects on the liver. Various changes in the liver parenchyma with remarkable fatty change in two cases in this series have been noted, but we observed only a slight infiltration of round cells with usually no accompanying increase of connective tissue in the interlobular spaces. The cases in which we injected extract of cigar tobacco repeatedly into the ear vein showed almost analogous changes, though they were not quite so marked. The difference again may be a difference in concentration. Thus the results of these experiments with nicotine were quite different from those of Laennec's cirrhosis of the liver. We may say, therefore, that there is no special relation between cirrhosis of the liver and nicotine, as far as these experiments go.

3. Through the repeated injections of sterilized emulsion of human tubercle bacilli into the portal vein and mesenteric veins of the rabbit, there followed marked changes resembling tuberculous lesions which were followed by an increase of connective tissue, chiefly in the interlobular spaces, together with some toxic effects on the parenchyma of the liver. These experiments seem to indicate, then, that cirrhotic changes in

the liver may be produced by killed tubercle bacilli and their toxic products without the presence of any living tubercle bacilli, and that there may be some relation between tuberculosis, especially of the peritoneum and intestine, and cirrhosis of the liver.

4. The series of cases in which was injected an emulsion of killed colon bacilli repeatedly, into the portal and mesenteric veins, showed marked cell infiltration and some proliferation of connective tissue in the interlobular spaces and some toxic effects on the parenchyma of the liver. Thus it is also evident that sterilized colon bacilli and their toxic products may produce cirrhotic changes in the liver, though not the same as Laennec's cirrhosis.

5. In spite of the marked cirrhotic changes of the liver in some cases, no ascites has been seen in these experiments, and it is probably very difficult to produce ascites under such experiments.

6. The extent of the changes in the liver is not always proportional to the frequency of the injections of toxic substances into the portal or mesenteric veins in the same rabbit or in different rabbits, as was pointed out above. In the latter it may depend more or less on the individuality of the rabbit, but the results obtained may be more reasonably explained by the observation that the cells which were once injured by the toxic substances are regenerated and then develop an increased resistance to these substances.

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DESCRIPTION OF PLATE IV.

FIG. I. (Table I., Case 2). — Marked increase of connective tissue and cell infiltration in the interlobular spaces and about central veins.

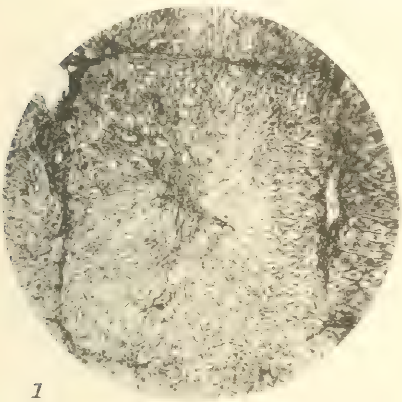
FIG. II. (Table II., Case 1). — Moderate cell infiltration, with slight proliferation of connective tissue in the interlobular spaces.

FIG. III. (Table III., Case 7). — Moderate cell infiltration in the interlobular spaces, with no apparent proliferation of connective tissue.

FIG. IV. (Table IV., Case 2). — Cell infiltration in the interlobular spaces, without any proliferation of connective tissue.

FIG. V. (Table V., Case 3). — Tuberculous lesions chiefly in the interlobular spaces, where there is some proliferation of connective tissue.

FIG. VI. (Table VI., Case 2). — Marked cell infiltration in the interlobular spaces, with slight proliferation of connective tissue and many vacuolated liver cells at the periphery of the lobules.



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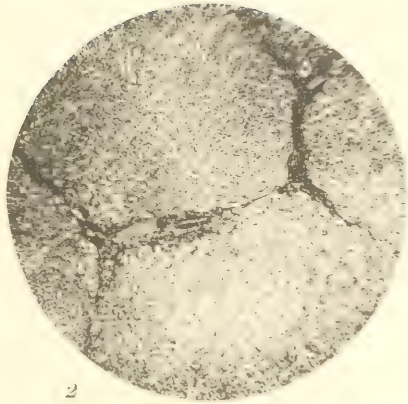

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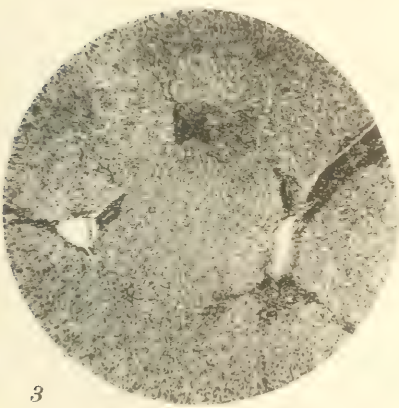

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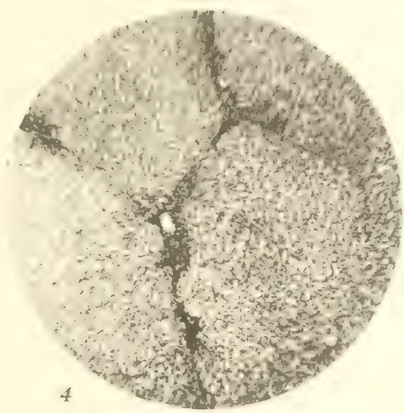

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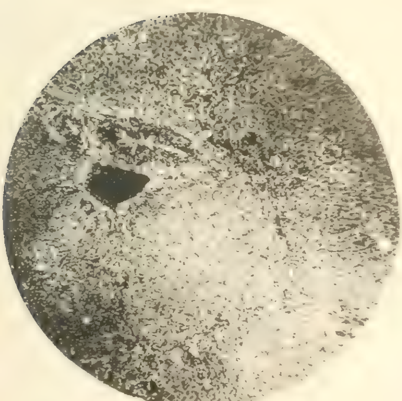

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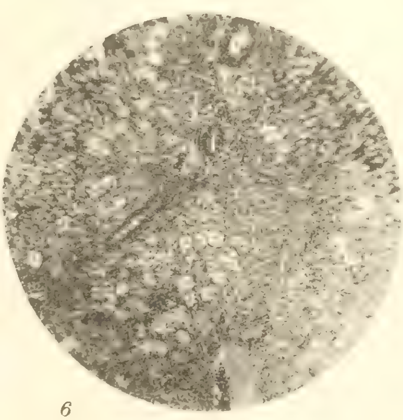

6

Ogata.

Cirrhosis of Liver

PRELIMINARY REPORT OF STUDIES ON THE INFLUENCE OF ALCOHOL AND NICOTINE UPON THE OVARY.*

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It has been maintained by many authors that chronic alcoholism of the parents has much influence upon the descendants. Simmonds[1] found atrophy in sixty per cent of the testicles of alcoholic adults examined at autopsy, while Cordes[2] observed no atrophy of the testicle in alcoholics at autopsy. Bertholet[3] found an atrophy of the testicle in numerous autopsies of chronic alcoholics. Forel[4] discussed blastophthoria in alcoholism from the standpoint of statistics. Iwanow[5] investigated the direct influence of alcohol upon the spermatozoa — performing artificial gestation experiments — and concluded that the direct influence of alcohol upon the spermatozoön is much less than the indirect influence of alcohol when introduced into the body. This may be explained in that fully developed spermatozoa are influenced by alcohol to a less degree than they are when still in the process of spermatogenesis. Kyrle[6] made an experimental study on the influence of alcohol upon the testicle and observed atrophy and other changes in the testicle. Arlitt and Wells[7] found in rats an almost constant change in the testes, consisting mainly in alterations in spermatogenesis.

While there are many investigators who have observed atrophy and other changes in the testicle together with injurious effects in the descendants as a result of experiments and human necropsy and also statistics, I have not found many authors who have studied the influence of alcohol upon the ovary. Arlitt and Wells, incidentally to their study of the testes of rats, investigated the ovaries of alcoholized female rats and found the ovaries sometimes atrophic and sometimes

* Received for publication May 7, 1919.

apparently poor in ova. But no definite conclusions were drawn. To be sure, the ovum has half the responsibility in reference to hereditary characters in the offspring. Then, further, there are many women who drink a great deal of alcohol at one time and are often even in a condition of chronic alcoholism. Tobacco also is used most widely, and it may produce some changes in different organs and in the walls of blood vessels. Therefore I studied here also the influence of nicotine upon the ovary.

I used relatively young rabbits for the experiments, but took some that were sexually fully developed. Three to five days before the first injection I always performed an ovariectomy, removing the left ovary in every rabbit in order to compare with the final findings. These ovaries which were removed were all normal, having various sizes and each weighing between .32 and .06 grams.

Alcohol experiments. — A solution of forty per cent alcohol was prepared by adding sterilized physiological salt solution to absolute alcohol. Six rabbits were injected by way of the ear vein once every two to four days with gradually increasing doses from four cubic centimeters to eight cubic centimeters. I killed the rabbits usually two to three days after the last injection.

No. of Rabbit.	Total Period of Injection in Days.	Total Number of Injections.	Total Quantity of Alcohol Used.	Remarks.
1	76	20	123 cc.	Weighs less than normal ovary. Fewer developing Graafian follicles than in the normal ovary.
2	62	16	92 cc.	Ditto.
3	60	17	96 cc.	Ditto.
4	43	11	65 cc.	Heavier than normal. Development of Graafian follicles normal.
5	31	9	48 cc.	Weighs less than normal. Development of Graafian follicles normal.

In these experiments we see no particular changes in the ovary macroscopically. The weight of each ovary is usually a little less than that of the normal excised ovary. It is evident that the comparison of weight has not much value in determining hypertrophy or atrophy of the ovary, because the ovary is such a changeable organ under normal conditions; but if we consider the compensatory hypertrophy and the development of the ovary in growing animals, together with the fact that the right ovary is usually heavier than the left, it may be considered that in the above cases there is more or less atrophy of the ovary or at least an inhibition of development.

Into the same cage with two rabbits after a period of injections of forty days (quantity of alcohol used, fifty-eight cubic centimeters), was put a normal male rabbit, but the females were not pregnant when killed.

Corresponding sections were made from the normal and pathological ovaries, but no particular changes were found in the structure of the Graafian follicles and stroma of the latter when compared to the former. The developing Graafian follicles in three cases, however, seemed not to be as numerous as in the normal ones, though in the other two we could not find any difference. The development of follicles may change in normal conditions especially in the period of rut, but in these three cases in which the alcohol was injected many times over a long period of time we may consider that the alcohol had some influence on the reduced number of developing Graafian follicles. In short, we could not find any apparent changes in the ovaries with repeated alcohol injections into the ear vein, though in some cases there were fewer developing Graafian follicles than in the normal in addition to the inhibition of growth of the ovary.

Nicotine experiments. — An extract of tobacco was prepared by adding fifty cubic centimeters physiological salt solution to one gram of a certain brand of cigar tobacco; after leaving it twenty-four hours at room temperature it was filtered through a Berkefeld filter. Four rabbits were injected with the filtrate by way of the ear vein once every two to four

days, with gradually increasing doses from three to five cubic centimeters. They were killed two to three days after the last injection.

No. of Rabbit.	Total Period of Injection in Days.	Total Number of Injections.	Total Quantity of Extract Used.	Remarks.
1	64	22	62 cc.	Weighs less than normal ovary. Pregnant.
2	53	17	48 cc.	Heavier than normal.
3	48	14	34 cc.	Heavier than normal.
4	35	11	29 cc.	Heavier than normal.

Macroscopically, there are no particular changes in the ovaries, except in one in which we see a quite recent bursting of the Graafian follicle. In comparing the weight of the ovaries we find in one case the pathological ovary is just a little lighter than the normal excised ovary while in the others the pathological ovaries are all heavier. One of the two rabbits which lived in the same cage with the normal male rabbit for forty days after the injections had started, was pregnant, but we cannot say here whether the young would have been physically and mentally healthy or not.

Microscopic examination reveals no particular changes; that is, there is no apparent reduction in the number of developing Graafian follicles as we saw in some of the cases where alcohol was employed. Further, we could not find any apparent changes in the structure of the Graafian follicles and the stroma of the ovaries. Thus we observe no apparent changes in the ovary in these experiments either microscopically or macroscopically.

Further work on this subject is planned, which will be an investigation of the offspring of female animals treated with alcohol and nicotine.

The ultramicroscopic changes of the ovary as a result of the injections of alcohol and nicotine we cannot at present discuss, but if we consider the injurious influence of alcohol upon the

testicle, which was recognized by many authors, together with the above experiments, we may come to the following conclusion: The fact that chronic alcoholism of parents has a great influence on their descendants seems to be principally dependent on the injurious influence of the alcohol on the spermatozoön, though in a less degree it may depend on the influence of alcohol upon the ovary. We may say also that, so far as our experiments show, there is no apparent influence of nicotine on the ovary.

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THE EFFECTS OF X-RAY IRRADIATION ON LIVING CARCINOMA AND SARCOMA CELLS IN TISSUE CULTURES IN VITRO[1]

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During the years which have passed since the method of tissue culture in vitro was commenced by Harrison, and modified by Carrel and Burrows (4), very little attention has been paid the investigation of the biological effects of x-rays upon cells growing in culture in vitro.

Contamin (7) exposed mouse carcinoma to x-rays under various experimental conditions. From the result of a number of his observations, he came to the conclusion that the younger the tumors, the more sensitive they were to x-rays, and that the disappearance of a large tumor under x-rays caused the death of the animal, probably by intoxication. In other experiments made by Contamin (18) with Nogier and Jaubert de Beaujeu, extirpated mouse carcinoma was exposed to x-rays and then inoculated into normal mice. They concluded that the action of x-rays on extirpated tumor cells hinders their subsequent growth in the animal body.

Clunet (5) and Raulot-Lapointe treated malignant tumors in situ with x-rays and studied them histologically at various stages of the treatment. They found that the squamous carcinoma cells in the human subject finally disappeared, passing through five successive stages from the latent phase to the formation of the connective tissue scar. With sarcomatous growths, Clunet and Raulot-Lapointe found that the latent phase was much shorter than in the other types of malignant growths.

[1] The author has not read the proof of this article.

Wedd and Russ (31) reported a series of experiments in which a transplantable mouse carcinoma was removed from the animal in which it had grown, kept between mica sheets during the exposure to radium rays, and then inoculated into normal mice. It was found that no growth resulted from grafts which were exposed a sufficient length of time.

Russ and H. Chambers (25) reported an observation made with Jensen's sarcoma. The authors concluded that the tumor cells irradiated by β-rays of radium (1.63 mgm. per square centimeter for ninety-six minutes) or radium emanation of 0.53 millicuries per cubic centimeter for forty-five minutes, did not produce tumors after inoculation into normal mice, though they showed no histological changes in the cells.

Wassermann (30) exposed extirpated cancerous tissue to radium rays from mesothorium, and then inoculated it into animals, no tumor resulting. He supposed that the multiplication and cell division were affected by the rays while the nutrition of the cells remained uninfluenced, though he did not try cultures in vitro.

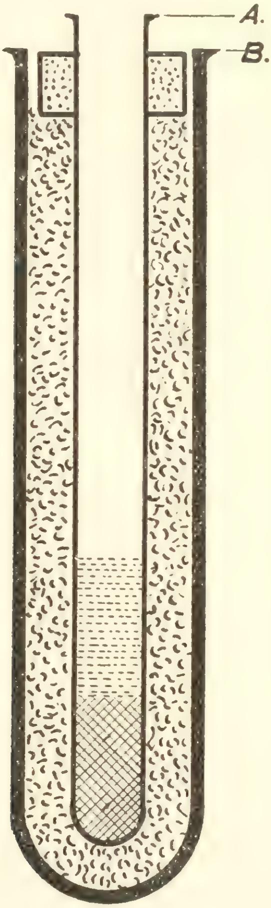
C. Price Jones (12) exposed mouse carcinoma and mouse sarcoma to radium rays, made tissue cultures of them, and found that the mitotic division of cells was inhibited, though their spreading growth was not retarded.

Prime (23) has recently worked in the same direction, reporting that a carefully measured amount of radium rays injured the power of mitotic cell division in the tissue, and that the inoculation of a sufficiently irradiated tumor produced no growths in mice.

In all these articles, however, no observations seem to have been made on the effects of x-rays upon living cells growing in plasma outside of the body. The present experiments were undertaken, therefore, to clear up the question of the biological effect of x-rays on tumor cells, and to determine what dose of x-rays would render tumor cells incapable of producing tumors when subsequently inoculated into animals.

PREPARATION OF THE CULTURE MEDIA

A small glass tube (*A* in text-fig. 1) was coated thickly with paraffin and inserted into an aluminum centrifuge tube (*B* in



Text fig. 1. Tube in which plasma is obtained.

text-fig. 1) stopped by means of a cork. The space between tubes *A* and *B* was filled with ice and salt mixture, and the whole apparatus kept in a glass with ice mixture until ready for use.

Under ether anesthesia the carotid artery of a guinea-pig was exposed about 2 to 3 cm. The distal part was ligatured by a thread while the proximal part was fixed by a small artery clamp to stop the blood stream. Proximal to the ligature the artery was pinched by a small clamp without narrowing the lumen.

Between this and the ligature the artery was cut off sharply. After the proximal clamp had been released, the blood was allowed to flow directly into the tube *A*, which was kept cool in ice mixture. When the blood was filled up to one-third the height of the tube *A*, the artery was clamped again. The tube with blood was promptly and powerfully centrifugalized for three minutes. The supernatant plasma (text-fig. 1) was separated by a small pipette into four or five small tubes, which were coated thickly with paraffin and prepared in ice mixture. It was necessary neither to keep the pipette cool nor to coat it with paraffin. These tubes which held the plasma were plugged with sterilized cotton and covered with tinfoil in order to protect them from contamination or drying, and preserved in a frozen condition in a Universal jar filled with ice mixture. The greatest difficulties encountered were obtaining a sufficient amount of mouse blood and keeping the plasma from coagulating. The mouse was prepared by the removal of the hair from the throat region, and the skin rendered sterile by iodin. The skin over the thyroid region was picked up with a forceps and cut off with scissors. The thyroid gland was picked up and bluntly loosened from the under layer without injuring the tissues. The carotid artery appeared on both sides in the bottom of the space made by picking up the gland. After the artery had been cut, the escaping blood was collected in the paraffin coated tube (text fig. 1) in ice mixture. In the same manner as described already, the tube was immediately centrifugalized for three minutes. The supernatant plasma was drawn up and put into cold tubes in ice mixture.

It was found very easy to obtain chicken plasma. A heavy syringe needle, sterilized by boiling, was inserted directly into the wing vein and the blood was collected drop by drop in an ice cold

paraffin coated tube, and centrifugalized in the same way as above. The supernatant plasma was drawn off into tubes.

The plasma thus obtained from guinea-pigs, mice, and chickens, did not lose its coagulability for about ten to fourteen days as it was kept in ice mixture, and remained fluid for hours during each series of experiments, when it was kept cool in ice, and at no time did coagulation take place before the completion of the experiment. One tube containing 0.2 to 0.3 cc. of plasma was enough for each series of experiments.

MATERIAL AND TECHNIQUE OF CULTURE

The tumors used were mouse carcinoma R.T. 33, which had been propagated for three years at the State Institute for the Study of Malignant Disease, Buffalo, New York. These tumors, for which I am indebted to Dr. Gaylord, showed a type of adenocarcinoma (fig. 2) and with the mice which I had inoculated gave "takes" in about 90 per cent of implants. Another tumor, for which I am indebted to Dr. F. C. Wood of the Crocker Fund, was the Ehrlich mouse sarcoma (fig. 3), which had been under observation for some years in the Crocker Laboratory in New York, and gave approximately 100 per cent of successful inoculations in Chicago mice.

The tumors, after reaching a full growth, were cut out strictly aseptically in Ringer's solution and cut into many pieces of equal size. They were then put into three or four small sterilized glass tubes with a diameter of 1 cc. and 1.5 cc. in depth. Each of these glass tubes was stopped with a cork pushed in over a sheet of sterile Japanese paper in order to prevent any contamination that might occur during the exposure of the x-rays. After the removal of the cork stopper, the pieces of tumor, which were kept in these tubes with Ringer's solution, were exposed to x-rays merely through the layer of Japanese paper at a distance of 2 cm. from the x-ray bulb. In this manner each piece of tumor was exposed for a certain required length of time without other obstacles between the bulb and the tumor pieces. Another factor in favor of this method was the fact that the tissues did

not begin to grow while exposed to the ray as was found to be the case when they were planted first into the plasma and then exposed.

The ray used for this series of experiments was of a moderately soft type, the spark gaps ranging from 4 to 8 cm.; the length of time for exposure varied from five to thirty-six minutes. The amount of the effective ray was measured by Hampson's radiometer. The initial tint is the color of the unexposed pastille and the sixteenth change represents the browner shade of color, equivalent to the maximum or B tint of the Sabouraud's pastille. The terms E.1, E.4, E.8, or E.12 in the following experiments indicate that the Hampson's pastille used showed no. 1, no. 4, no. 8 or no. 12 tint, that is, equivalent to a dose $\frac{1}{16}$, $\frac{1}{4}$, $\frac{1}{2}$, or $\frac{3}{4}$ of Sabouraud's B tint.

The irradiated tissue was removed to a watch glass with Ringer's solution and was cut into fine fragments. Each fragment was transferred to a cover glass by a small pipette provided with suction bulb. The excess Ringer's solution was sucked up with the same pipette. Some small drops of plasma were added immeiately to the tissue fragment, then some drops of mouse serum with Ringer's solution were added. The plasma and serum were mixed up and spread around the fragment with a pointed cataract knife. The cover slip, which previously was ringed with vaseline on all its edges, was then inverted over a fairly deep hollow ground slide. The cover slip was then sealed with molten paraffin around its edges. An equal number of control cultures from an irradiated tissue was made in each series. The slide preparations were incubated at 37°C. and microscopic observation was made every twelve to twenty-four hours.

To obtain the stained specimen the cover slips, mounted with growing culture, were put into 10 per cent formalin for twenty-four hours or more, then they were washed in water for one hour and stained with diluted Delafield's haematoxylin for one to two hours, and decolorized for about thirty minutes in water, to which a few drops of diluted hydrochloric acid was added. The specimens were washed again in water until a violet blue color developed. It is important to decolorize the over-stained plasma and

tissue as much as possible, otherwise no good specimen was obtained.

The tissue piece irradiated with certain required doses of x-ray in Ringer's solution was divided into many fragments, each of about 20 mgm., and these were inoculated into the right axilla of a number of normal mice. An equal number of tissue fragments from the control tissue were inoculated into the other axilla of the same mice. The observations respecting the rate and course of the grafts planted into the animals follows.

The culture media used for the experiments were: Mouse plasma, guinea-pig plasma, chicken plasma, guinea-pig plasma plus mouse serum diluted with Ringer's fluid, chicken plasma plus mouse serum diluted with Ringer's fluid.

In comparing all these media used for the culture of the tumor, it was found that the mouse plasma (homogenous and autogenous) or a mixture of guinea-pig plasma and mouse serum diluted with Ringer's solution were most satisfactory as media for the culture of the tumors. There was no noticeable difference in either case, whether the mouse plasma or the guinea-pig plasma with diluted mouse serum was used. Because the guinea-pig plasma can be obtained much more easily and in greater quantity than mouse plasma, most of the cultures, except a few specimens, were made with guinea-pig plasma, to which was added the mouse serum and Ringer's solution.

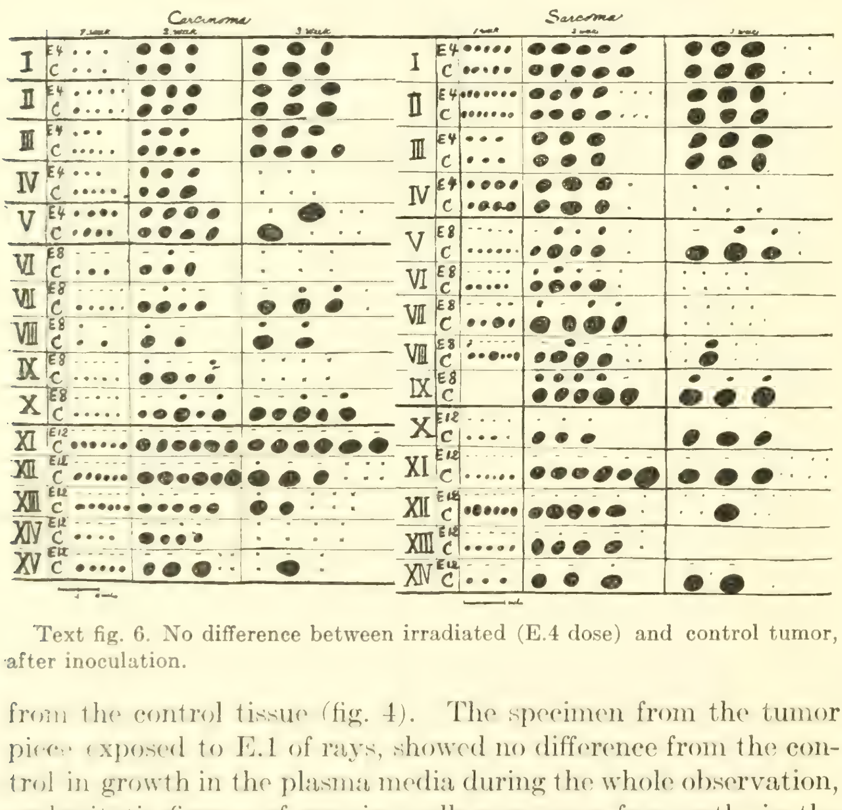
RESULTS OF EXPERIMENTS

Mouse carcinoma

Mouse carcinoma, cultivated under the conditions described above in respect to technique, showed but very little growth. the first twenty-four hours in most cases of all series, both in the control and in the exposed tissue. After twenty-four hours of incubation, the original fragment of tissue became thinner and somewhat translucent, especially on the edge of the fragments, from which the cells spread out into the plasma media. The cells did not migrate separately into the media, but merely formed cell groups composed of a number of cells. The boundaries of each cell were indistinct.

Many of the cells on the advancing edge formed pseudopodia. There were many cells of different morphological type which supposedly originated from the stroma. Cells of this type were present in some specimens, while others showed no such type of cells.

Most cultures reached the maximum growth in forty-eight to ninety-six hours after incubation. Karyokinetic figures were observed in the cells in the growing zone in stained specimens



Text fig. 6. No difference between irradiated (E.4 dose) and control tumor, after inoculation.

from the control tissue (fig. 4). The specimen from the tumor piece exposed to E.1 of rays, showed no difference from the control in growth in the plasma media during the whole observation, and mitotic figures of growing cells were seen frequently in the stained preparations in all stages. Both the tissues, control and irradiated, produced good sized tumors after inoculation into normal mice.

In cultures from the tissue exposed to E.4 of the ray, the proliferation of cells was just as extensive as in the specimen made from control tissue (figs. 9, 10), while the spreading-out of the thread-like cells, which supposedly originated from stroma, showed itself a little more active than in the control. The mitotic figures were found as abundant as in control cultures (fig. 5).

No difference was seen in speed and rate of growing tumors, produced by inoculation of either the treated or the control piece, as is shown by the preceding chart (text-fig. 6) and by table 1.

TABLE 1

Carcinoma

	NUMBER OF "TAKES"		PER CENT	NUMBER OF "TAKES"		PER CENT	NUMBER OF "TAKES"		PER CENT
	E.4	Control		E.8	Control		E.12	Control	
First week	19	19	100	3	19	16.0	0	26	0
Second week	16	16	100	8	18	44.5	0	24	0
Third week	10	10	100	6	10	60.0	0	12	0

In the unstained culture, when E.8 doses of x-ray were given, the proliferation of cells growing in groups showed almost the same extension as did the specimen of control tissue (fig. 11). In some specimens, however, the cells of the stroma type, which spread out like fibrillae into the plasma media, seemed more vigorous than those in the control specimens. But in the stained specimen a few mitotic figures were found in most of the specimens cultivated from the irradiated tissue (fig. 7), while many more mitotic figures appeared in the control specimen (fig. 4). After the inoculation into mice, it was found that the treated tissue gave "takes" of 16 per cent in the first, 44.5 per cent in the second, and 60 per cent in the third week, according to table I, while the control tissue produced tumors in 100 per cent of the grafts.

All the tumors grown in mice from the treated cells, however, reached only one-third of the size of those from the control inoculations.

In the cultures of the tissue which was exposed to E.12 of the active ray, it was found that after forty-eight hours there was still a marked outgrowth of cells (fig. 12). The same results were found in the control specimen. None of the stained specimens of the exposed tissues showed mitotic figures in growing cells (fig. 8).

The inoculation of the treated tissue into mice produced in the first week no tumor in 27 cases in 6 series. In the second and third weeks two nodules were developed to the size of a rice grain; they, however, did not grow further, but disappeared.

Mouse sarcoma

In the cultures made in guinea-pig plasma diluted with mouse serum, the margin of the fragment became gradually sharp and opaque. In five to twelve hours a few round cells had emigrated into the plasma media and a few irregular cells began to grow out radially from the edges of the fragment, which began to have a serrated appearance. The individual cells formed many pseudopodia, which were seen especially in the advancing side of the cell body. The number of cells emigrating into the media increased more rapidly than those in the culture of carcinoma tissue. After forty-eight hours the original fragment became more translucent than it was before, and it was surrounded with thick layers of growing cells, which were rich in protoplasm. When the stained specimens were fixed, at the end of twenty-four to seventy-two hours, they exhibited the presence of numerous mitotic figures in the outgrowing cells (fig. 13).

The specimen from the tissue exposed to E.1 of x-ray showed no difference in respect to the rate of growth from that of the control tissue (fig. 17). The inoculation of the treated piece into mice produced tumors as large as those of the control tissue.

In the culture of the tissue exposed to E.4, the proliferation of cells was found to be more vigorous than that in the control cultures (fig. 18). In the stained preparations, however, the number of mitotic figures of cells was about equal to that in the control specimen (fig. 14). After inoculation into mice, the

tumors produced from irradiated cells in the first week seemed to grow a little faster than those in controls; but in the second and the third week there was no difference either in speed or size, and the inoculation gave "takes" of 100 per cent.

In the unstained cultures from the tissue which was exposed to E.8, the proliferation of cells and the area of growth were similar to the proliferation in the control specimen (fig. 19). But the mitotic figures in the stained specimen diminished greatly in number compared with those in the control specimen (fig. 15). In the first week after inoculation, the grafts gave "takes" of 35 per cent, 62 per cent in the second, and 85.7 per cent in the third week. These tumors, however, developed only to one-third the size of those in the controls (text-fig. 6).

TABLE 2

Sarcoma

	NUMBER OF "TAKES"		PER CENT	NUMBER OF "TAKES"		PER CENT	NUMBER OF "TAKES"		PER CENT
	E.4	Control		E.8	Control		E.12	Control	
First week	19	19	100	7	20	35.0	0	24	0
Second week	15	15	100	10	16	62.5	0	21	0
Third week	9	9	100	6	7	85.7	0	9	0

In the cultures made from the tissue which had been irradiated to E.12, it was found that there was still a marked outgrowth of cells as well as in the control specimen (fig. 20). No mitotic figures, however, were found in the stained specimen (fig. 16). None of the inoculated grafts into 23 mice in 5 series produced any tumor the first week. In the second week there were found 4 hardly perceptible nodules in 21 mice. These, however, did not grow further and disappeared entirely in the third week, as is shown in text-fig. 6 and table 2.

DISCUSSION

In accordance with the results of the experiments described above, the rate of the cell growth in cultures of mouse carcinoma and mouse sarcoma was, after forty-eight to ninety-six hours'

incubation, nearly equal in both the control and the exposed tissues which were x-rayed to E.1–E.12 (figs. 9, 10, 11, 12, 17, 18, 19, 20). The sarcoma, as compared with the carcinoma, however, was always superior in its growth in culture media. After an exposure of E.8, the number of the mitotic figures in the culture of growing tumor cells was diminished to a minimum of 2 to 4 in carcinoma and 2 to 13 in sarcoma cultures. An exposure, however, of E.12, entirely inhibited the mitotic division of cells, and they were never found in the stained specimen, either in carcinoma or in sarcoma (figs. 8, 16). See table 3.

By an exposure of E.4, the sarcoma not only remained without injury to the power of proliferation by mitotic cell division, but the exposed tissue produced tumors in the first week after inoculation —i.e., somewhat earlier than the control tissue did (text-fig. 6). This phenomenon was due to the action of x-rays, to which the tissue was exposed. It seems that the x-ray in this dose acted upon the tissue as a stimulation and temporarily raised the metabolism of the tumor cells. Consequently the cells in the exposed tissue were stimulated to grow more quickly the first week after inoculation than those in the control.

In connection with the process of oxidation in the tumor tissue, I tried some experiments hoping that the effects of x-ray on the living cells might be explained to some extent. The tumor pieces were exposed to x-ray of various doses varying from E.4 to E.12. One piece of control was put in the chamber of one side of Dr. Tashiro's biometer and an irradiated piece in the other chamber of the other side.

Observation was made as to the quantitative difference of CO_2 production in both chambers. The results of these experiments are shown in the record of tables 4 and 5.

In the experiments with the tumor pieces, both in carcinoma and sarcoma, which were exposed to E.4 of x-ray, carbon dioxide production began to appear in ten minutes after the arrangement. The quantity of precipitation by barium hydroxide in the chamber which contained the exposed tissue was greater than that in the chamber which had the control tissue.

TABLE 3

SPECIMEN	NUMBER OF FIGURES	AMOUNT OF X-RAY	LENGTH OF TIME	STAINED SPECIMEN	
				Growth in culture	Number of mitotic figures
Carcinoma					
			minutes		
111	4, 9	Control		Fair	7
4				Fair	15
31				Slight	8
102				Slight	16
110	10, 5	E.4	12	Fair	6
104				Fair	8
101				Slight	4
103				Slight	7
18	11	E.8	24	Fair	2
100				Slight	3
35				Fair	4
28				Slight	4
5	8, 12	E.12	36	Fair	0
33				Slight	0
34				Slight	0
107				Fair	0
Sarcoma					
1	13	Control		Fair	21
95				Fair	40
105				Fair	Numerous
106				Fair	Numerous
43	14, 18	E.4	12	Fair	40
17				Fair	25
52				Fair	31
15				Fair	27
11	15, 19	E.8	24	Fair	10
98				Fair	6
94				Fair	13
50				Fair	2
96	16, 20	E.12	36	Fair	0
41				Fair	0
46				Fair	0
53				Fair	0

On the contrary, in the experiments made with the tissue exposed to E.12 of x-ray, the production of carbon dioxide was less than that of the control.

In the unstained specimen cultured from tissue which was exposed to E.4, the outgrowth of cells seemed somewhat more

TABLE 4

Carcinoma

CO_2 COMPARED				CO_2 COMPARED			
Time	E.12		Control	Time	E.4		Control
	*29 mgm		*27 mgm.		*75 mgm.		*75 mgm.
4.42	−−−−−−		−−−−−−	2.19	−−−−−−		−−−−−−
4.50	−−−−−−	=	−−−−−−	2.29	−−−−−−	>	−−−−−−
7.00	−−−−−−	<	−−−−−−	2.39	−−−−−−	>	−−−−−−
	++		+++	2.49	−−−−−−	>	−−−−−−
				2.59	+++−−	>	−−−−+
	52 mgm.		50 mgm.		75 mgm.		75 mgm.
7.50	−−−−−−	<	−−−−−−	2.35	−−−−−−		−−−−−−
7.55	−−−−−−	<	−−−−−−	2.45	−−−−−−	>	−−−−−−
8.00	−−−−−−	<	−−−−−−	2.55	−−−−−−	>	−−−−−−
8.10	−−−−−−	<	−−−−−−	3.05	−−−−−−	>	−−−−−−
				3.15	−−−−−−	>	−−−−−−
	++		+++	3.35	−−−−−−	>	−−−−−−
					++		+
	57 mgm.		55 mgm.		63 mgm.		64 mgm.
7.22	−−−−−−		−−−−−−	2.13	−−−−−−		−−−−−−
7.32	−−−−−−		−−−−−−	2.23	−−−−−−	>	−−−−−−
8.12	−−−−−−	<	−−−−−−	2.33	−−−−−−	>	−−−−−−
8.20	−−−−−−	<	−−−−−−	2.43	−−−−−−	>	−−−−−−
8.40	−−−−−−	<	−−−−−−	3.13	−−−−−−	>	−−−−−−
	++		+++		+++		++

* The figures are the weights of the pieces of tumor tissue used in each experiment. No quantitative determination of the amount of CO_2 produced was made, but the relative amounts produced by irradiated tumor and control are indicated by the > sign.

extensive than the outgrowth of the control culture, both in sarcoma and carcinoma. But in the stained specimens no noteworthy difference in number of the mitotic figures was seen. This stimulating effect on the sarcoma tissue was more prominent than the effect shown in the culture of the carcinoma. A some-

what similar difference of the stimulating effect of x-ray E.4 on the tissues appeared prominently in the inoculation experiments of sarcoma, but the carcinoma was not much stimulated by the rays and consequently the power of proliferation was diminished

TABLE 5

Sarcoma

CO_2 COMPARED				CO_2 COMPARED			
Time	E.12		Control	Time	E.4		Control
	*74 mgm.		*70 mgm.		*132 mgm.		*132 mgm.
2.25	------		------	3.43	------		------
2.40	------	=	------	3.53	------	>	------
2.45	------	<	------	4.03	------	>	------
3.00	------	<	------	4.43	------	>	------
3.10	------	<	------				
3.25	------	<	------		++++		+++
	++		+++				
	22 mgm.		16 mgm.		132 mgm.		132 mgm.
2.36	------		------	4.13	------		------
2.46	------	<	------	4.23	------	>	------
2.56	------	<	------	4.33	------	>	------
	+		++	5.13	------	>	------
					++++		+++
	48 mgm.		47 mgm.		85 mgm.		85 mgm.
3.38	------		------	6.33	------		------
3.48	------	<	------	6.43	------	>	------
3.58	------	<	------	6.53	------	>	------
4.08	------	<	------	7.03	------	>	------
4.18	------	<	------				
4.28	------	<	------		++		+
	+		+++				

* The figures are the weights of the pieces of tumor tissue used in each experiment. No quantitative determination of the amount of CO_2 produced was made, but the relative amounts produced by irradiated tumor and control are indicated by the > sign.

gradually without any preliminary stimulation (text-fig. 6 and table 4).

The fact that the number of mitotic figures in culture and the quickness of growth of the grafts inoculated into mice increased to some extent when the tissues previously were exposed to E.4

of rays, and the fact that both decreased gradually when the tissues were irradiated to E.8–E.12, coincide with the results of the experiments on the oxidation of the tumor tissues.

For the explanation of the presence of only a few mitotic figures in the culture and retardation of the growth of the grafts when the tissue was exposed to E.8, the reports made by Bordier and others are to be taken into consideration. We know that the younger the cell generation the greater the radio-sensibility of the living protoplasm; and consequently the younger neoplastic cells are most sensitive, while the cells in the latent stage are less sensitive to the x-ray action. Hence the mitotic figures seen in the tissues that had been exposed to E.8 supposedly originated from some resting cells in the fragments, in which they remained without an intensive effect of x-ray and produced the further division in culture in vitro or developed to a tumor in mice, though their mitotic proliferation was greatly retarded.

On the contrary, all the cells in the specimen exposed to E.12 were sufficiently damaged by the ray action, and consequently the dividing process of the cell chromosomes had ceased, and the fragments did not grow any more to a tumor in mice.

CONCLUSIONS

1. The mouse carcinoma and sarcoma grow as well in guinea-pig plasma to which has been added mouse serum diluted with Ringer's solution, as in mouse plasma itself.

2. The culture growths of carcinoma and sarcoma from mice showed each the characteristics of the original tissues. Sarcoma produced a radial outgrowth spreading widely into the plasma media, while the carcinoma cells grew continuously into the media, as cell groups, from the edges of the fragments.

3. The outspreading growth of cells in culture, both sarcoma and carcinoma, was not stopped by x-ray action varying from E.4 to E.12. The mitotic figures of cells were limited to a minimum after an exposure of E.8 (one-half dose of Sabouraud's B tint). After exposure to E.12 (three-fourth dose of Sabouraud's B tint), however, they disappeared entirely, and the treated tissue produced no tumor when inoculated into mice.

4. The growing power of sarcoma after E.4 exposure was stimulated to some extent, while carcinoma was not appreciably influenced. An exposure of tissues to E.12, both sarcoma and carcinoma, stopped the growing power of these tissues when inoculated into mice, and eliminated the process of mitotic division of cells.

5. The process of oxidation of tissues, both sarcoma and carcinoma, was stimulated by the *x*-ray action of E.4 and retarded by exposure to E.12 of the ray.

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PLATE 1

Fig. 2. Stained section of original carcinoma, many mitotic figures. × 275.

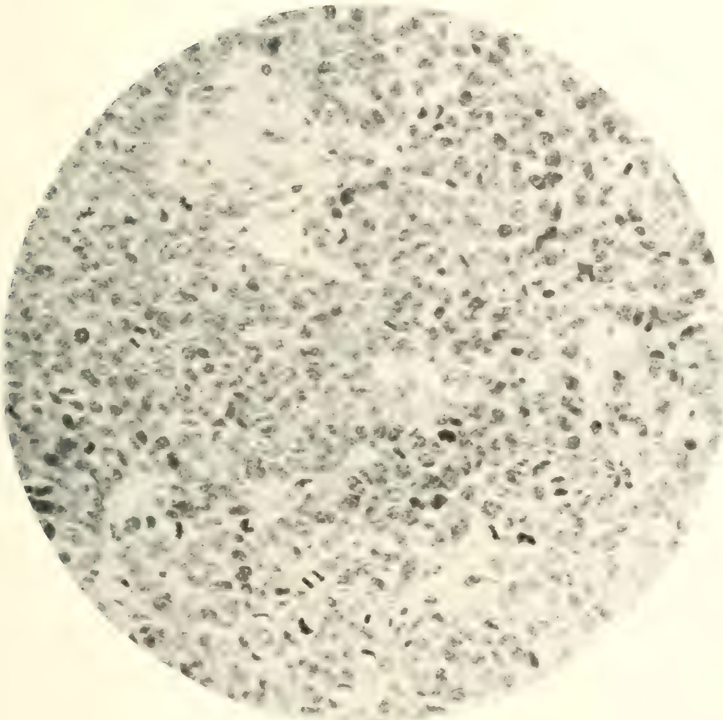


PLATE 2

Fig. 3. Stained section of original sarcoma, many mitotic figures. $\times$ 275.

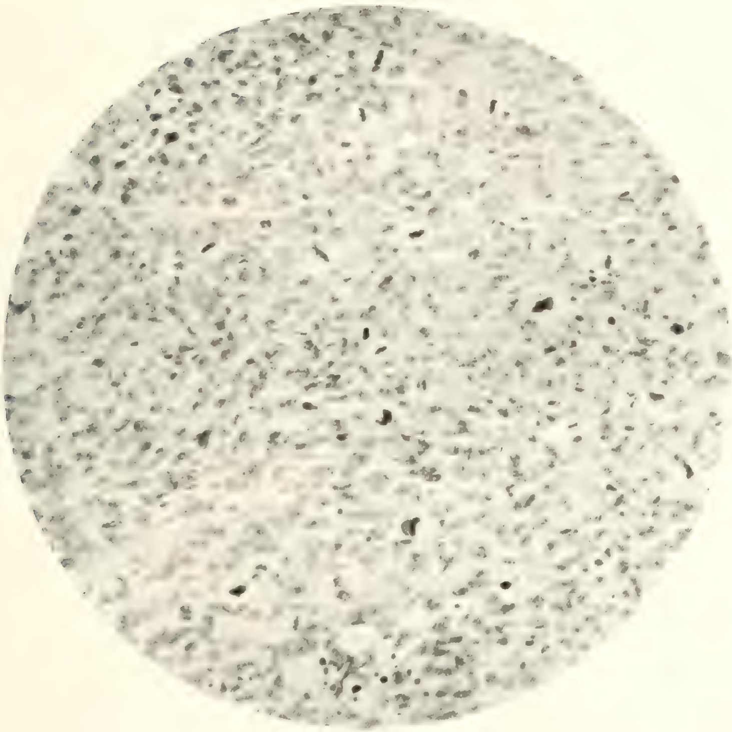


PLATE 5

Fig. 4. Tissue culture, control, carcinoma, 2 mitotic figures. × 275.

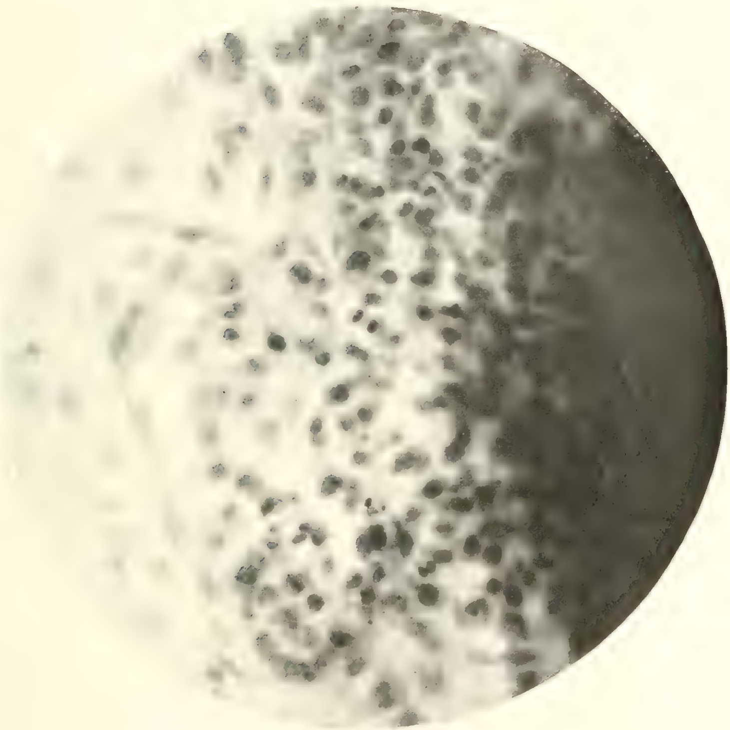


PLATE 4

Fig. 5. Tissue culture, experiment 4, carcinoma, 1 mitotic figure. × 275.

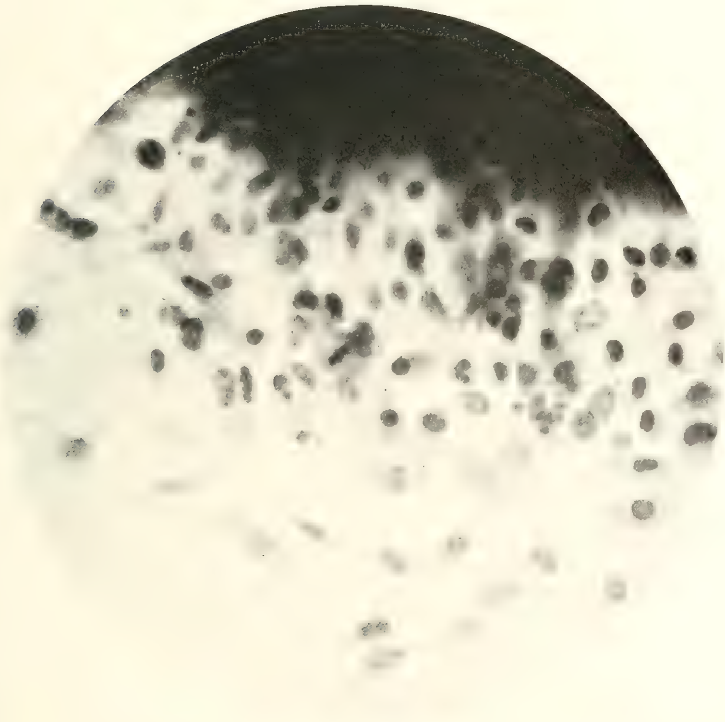


PLATE 5

Fig. 7. Tissue culture, experiment 8, carcinoma, 1 mitotic figure. $\times$ 275.

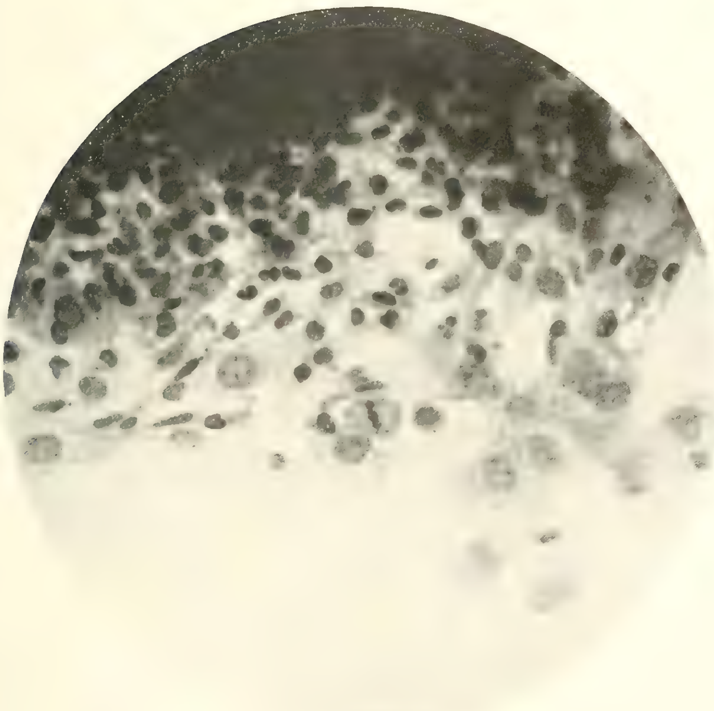


PLATE 6

Fig. 8. Tissue culture, experiment 12, carcinoma, no mitotic figure. $\times$ 275.

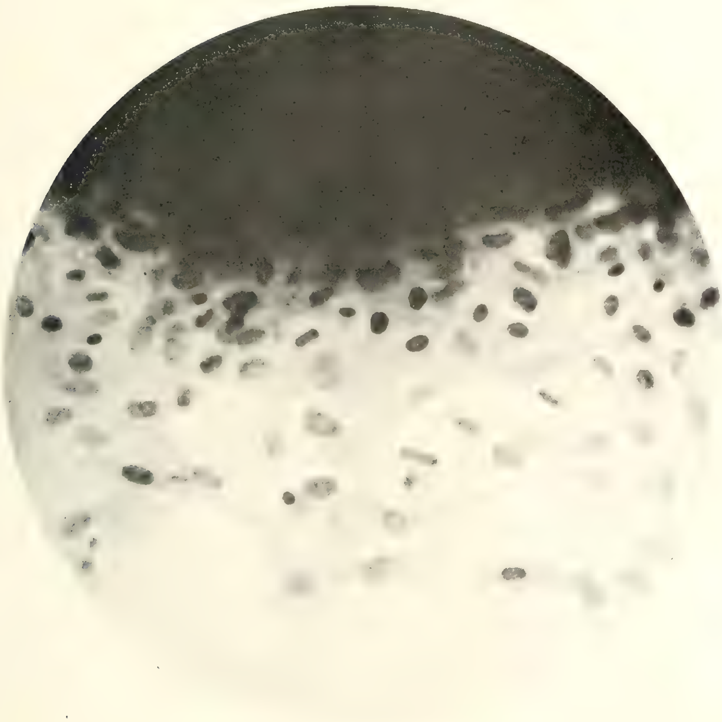


PLATE 7

Fig. 9. Tissue culture, control, carcinoma. × 37.

Fig. 10. Tissue culture, experiment 4, carcinoma. × 37.

Fig. 11. Tissue culture, experiment 8, carcinoma. × 37.

Fig. 12. Tissue culture, experiment 12, carcinoma. × 37.

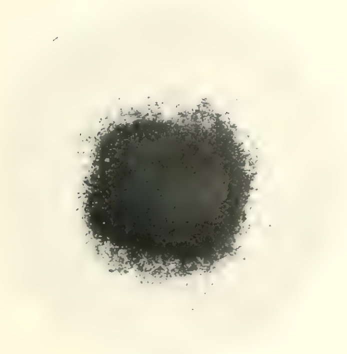


FIG. 9

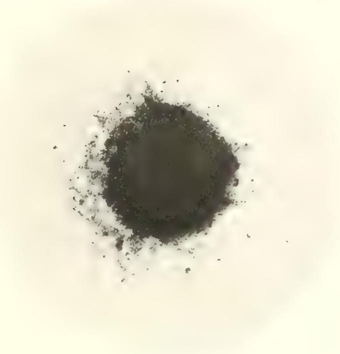


FIG. 10

FIG. 11

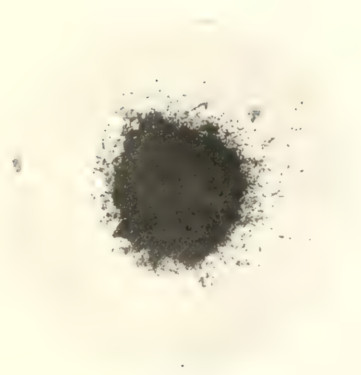


FIG. 12

PLATE 8

Fig. 13. Tissue culture, control, sarcoma, 2 mitotic figures. × 275.

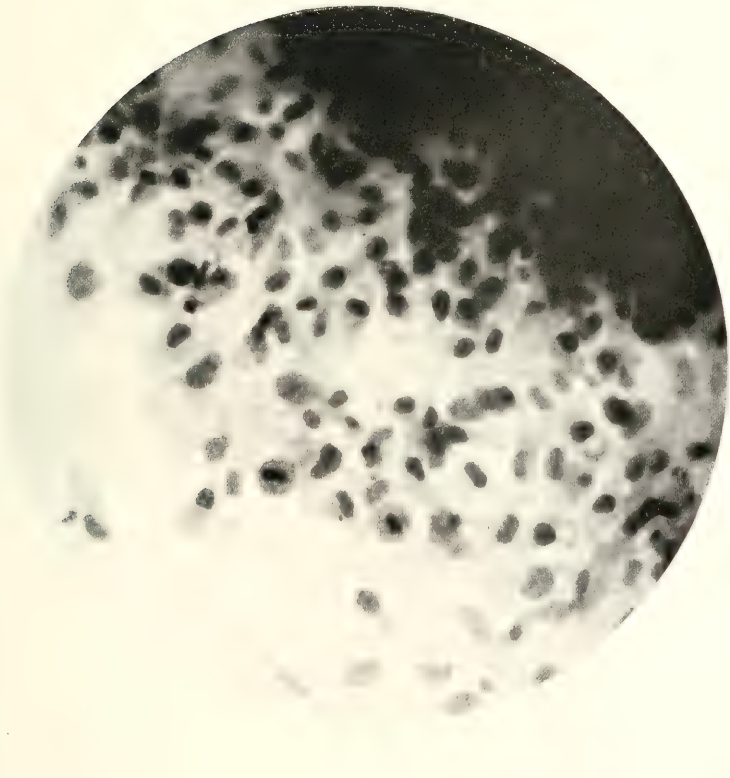


PLATE 9

Fig. 14. Tissue culture, experiment 4, sarcoma, 4 mitotic figures. $\times$ 275.

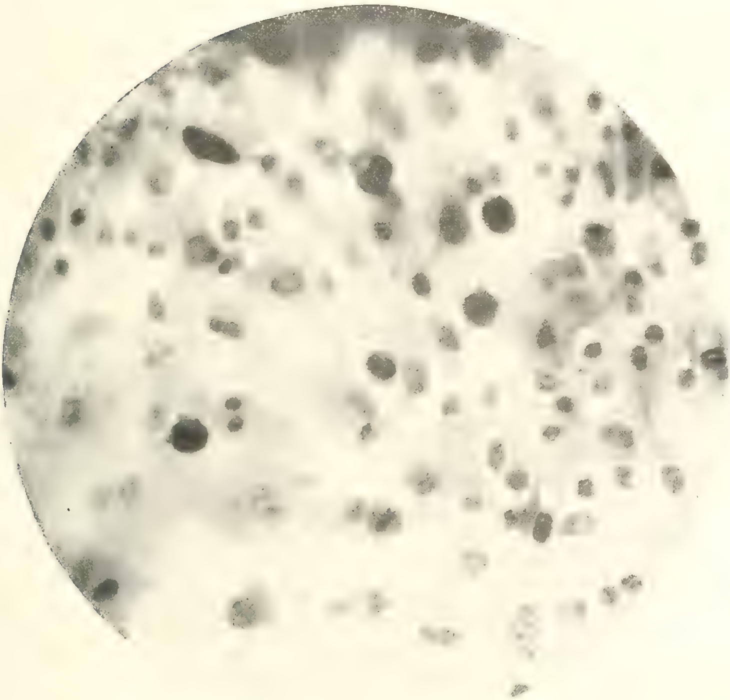
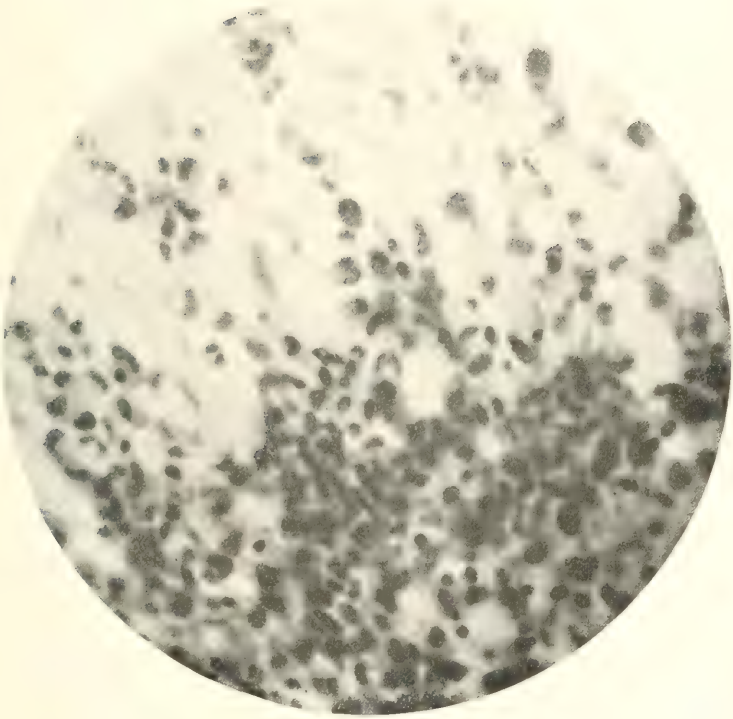


PLATE 11

Fig. 16. Tissue culture, experiment 12, sarcoma, no mitotic figure. $\times$ 275.



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THE ANTIGENIC PROPERTIES OF PROTEOSES

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INTRODUCTION

Of fundamental importance in immunity is the question whether only proteins are antigenic. Wells[1] says, "as yet, it has not been finally established that any colloids other than proteins can act as antigens." Assuming then, that only proteins possess antigenic properties, it becomes of interest to inquire what there is in the nature or constitution of proteins to which this is due. Two characteristics of proteins have received the greatest attention in this connection; their colloid nature and their huge molecular weight. Attempts to explain the antigenic properties of proteins on the basis of their colloid nature have been largely speculative and consist of the application of the principles of colloid chemistry to theories of immune reactions. The efforts to determine the influence of the size of the molecule on the antigenic properties of proteins have led to numerous attempts to produce antibodies for the products of protein hydrolysis and digestion.

Our understanding of the chemistry of immune reactions is limited by our knowledge of the chemistry of proteins. Each new insight into the nature and constitution of proteins is followed by a readjustment of our theories of immuno-chemistry. Underhill and Hendrix[2] place the discovery of the phenomenon of anaphylaxis among the more important recent advances in the physiology of proteins. It has also furnished considerable impetus to the study of protein chemistry.

For a satisfactory explanation of the anaphylaxis reaction, it became necessary to determine by what changes proteins were rendered toxic, since it was well known that native proteins were of themselves nontoxic. This led to the theories of Vaughan, Friedmann and Friedberger that anaphylaxis is due to parenteral digestion of proteins, and that the symptoms are due to intoxication with the resulting products. To obtain evidence in support of these theories the products of protein

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[1] Chemical Pathology, 1914, p. 156.

[2] Jour. Biol. Chem., 1915, 22, p. 443.

digestion and cleavage have been injected into animals in attempts to produce the symptoms of anaphylactic shock.

Considerable work has been done by many investigators in an effort to demonstrate the formation of antibodies against incomplete protein molecules. Most of the attempts have been made with the higher cleavage products of protein digestion.

HISTORICAL

(*a*) *Anaphylaxis and "Peptone Shock."*—Evidence is not lacking that the higher products of protein disintegration may act as antigens. The most successful results have been reported with the anaphylaxis reaction.

Rosenau and Anderson[3] were among the first to ascribe anaphylactogenic properties to "peptone." They state that "peptone" seems to have slight sensitizing and intoxicating properties but the table showing their results indicates that 0.004 gm. was injected subcutaneously into a guina-pig, followed 31 days later by a like amount which produced no symptoms of anaphylactic shock. They fail to mention the source or kind of "peptone" used.

Arthus[4] studied the anaphylaxis reactions of proteoses as represented by commercial Witte peptone. His first experiments were on dogs. He noted a striking resemblance between the reaction of sensitized animals to a second injection of serum and the reaction of normal dogs to an injection of "proteoses." He was able to increase the toxicity of Witte peptone by repeated subcutaneous injections; this he interprets as a hypersusceptibility to proteoses. Experiments with rabbits yielded practically the same results. In a sensitized rabbit (to horse serum) a dose of "peptone" which produced no effect in a normal animal caused a marked fall in blood pressure, dyspnea and loss of sphincter control.

It is doubtful if any of the reactions described by Arthus following the injection of Witte peptone represent true anaphylaxis reactions. Zunz[5] has pointed out that it is not at all surprising that conflicting results have been obtained by the use of Witte peptone, since its composition is extremely variable, especially the proteoses contained in a given sample. Much emphasis was laid on changes in respiratory rate in "sensitized" rabbits, whereas mere handling of these animals is often sufficient to produce marked dyspnea and tachycardia.

Pick and Yamanuchi,[6] using a 10% solution of Witte peptone from which all coagulable material had been removed by boiling, produced symptoms of anaphylaxis and death in a rabbit sensitized to beef serum. The dose of peptone was 4 c c intravenously. Two young rabbits passively sensitized against Witte peptone died on intravenous injection of 6 c c of 10% heated peptone. Control animals showed no symptoms. In these experiments young rabbits were used, and sensitization was passive.

After reviewing the symptoms of anaphylaxis in the different laboratory animals, Biedl and Kraus[7] called attention to the fact that the symptom complex as described in the literature up to that time was not at all definite.

[3] Hyg. Lab. Bull. 36, U. S. Pub. Health Serv., 1907.

[4] Arch. Internat. de Physiol., 1909, 7, p. 471; 1910, 9, p. 157 and p. 179.

[5] Ztschr. f. Immunitatsforsch., 1913, 16, p. 580.

[6] Ibid., 1909, 1, p. 676.

[7] Wien. klin. Wchnschr., 1909, 22, p. 363

They therefore made a careful study of anaphylaxis in the dog because they considered this animal most suitable for the study of physiologic phenomena. All of the dogs used did not respond to a second injection of horse serum. The characteristic symptoms in those that did react was a fall in blood pressure due to peripheral vasodilatation, a loss of coagulability of the blood and a primary leukopenia followed after several hours by a leukocytosis. None of their dogs died of an acute anaphylactic reaction. In comparing these symptoms with the action of Witte peptone, they noted a close similarity between the two. They concluded from their experiments and from review of the literature that the effect of peptone is in all respects similar to the reinjection of horse or beef serum even in minutest detail. They do not say that anaphylaxis is produced by peptones or proteoses but believe that Witte peptone contains the active principle which is responsible for the symptoms of anaphylaxis. As additional evidence they state that dogs were rendered antianaphylactic against serum by the injection of Witte peptone, and conversely animals which had recovered from the effects of a second injection of serum could withstand larger doses of peptone.

In a subsequent paper Biedl and Kraus[8] state that they were unable to confirm the observations of Arthus[4] that the symptoms of anaphylaxis in the rabbit are similar to those in the dog. They emphasize the fact that their experiments apply only to dogs. By the injection of cultures of various bacteria they were able to partly reproduce the symptoms of anaphylactic shock in normal dogs. These failed to appear when the cultures were grown on peptone-free medium. They therefore conclude that the symptoms were due to the small amounts of peptone present in the medium. Later the same authors[9] report the results of experiments on rabbits and guinea-pigs. In their opinion the mechanism of anaphylaxis is different in the dog and in the guinea-pig. The effect in the former is on the smooth muscle of the peripheral vessels leading to vasodilatation, while in the guinea-pig the reaction consists of a tetanic contraction of the smooth muscle of the bronchioles. Rabbits were found unsuited to anaphylaxis experiments because they died from blood pressure changes. Peptone proved to be very toxic for the guinea-pig, but the symptoms were not like those in the dog. After intravenous injection of 0.25 to 0.30 gm. Witte peptone into guinea-pigs they found that the interference with respiration and the physiologic and anatomic changes in the lungs were exactly like those in anaphylactic shock. From these results they conclude that in the guinea-pig as well as in the dog, Witte peptone has the same action as the toxic principle responsible for the symptoms of anaphylactic shock.

In discussing the results of Biedl and Kraus it may be said that altho they selected the dog as being most suitable for physiologic experimentation, anaphylaxis represents a special manifestation of immune phenomena, and it does not follow that an animal especially suited to work in the one field will also prove satisfactory in the other. As a matter of fact, the symptoms which they found characteristic of anaphylaxis in the dog do not support their contention. Changes in blood pressure may be produced by a number of chemical substances. The same is true of delayed coagulability of the blood. As Wells[10] points out, "the results obtained by observing changes in blood pressure in dogs are by no means comparable with results obtained

8 Cent. f. Bakteriol., 1909, 44, Ref. Beiheft, p. 68.

9 Ztschr. f. Immunitätsforsch., 1910, 7, p. 205.

10 Jour. Infect. Dis., 1909, 6, p. 506.

with guinea-pigs, on which most of the work so far reported has been done, since in these animals the symptoms are entirely different from the symptoms in the dog, and much more closely resemble the effects seen in man."

It is not the purpose to review in detail the physiologic properties of proteoses. This has been done by Chittenden, Mendel and Henderson.[11] They used purified albumoses obtained by acid hydrolysis and digestion of coagulated egg-albumin. All proteoses of whatever source, produced a fall in blood pressure which varied only in degree, and blood withdrawn even 1 or 2 hours after the injection failed to coagulate for at least 24 hours. To produce the characteristic effect 3 to 5 cg. per kg. of body weight are necessary and the intensity of the reaction depends much more on the rapidity of injection than on the dosage. An animal which has recovered from an injection of proteoses may fail to respond to a second injection. This has been termed "immunity" to proteoses, but might better be spoken of as a refractory state, the explanation for which is unknown.

Biedl and Kraus cite the experiments of Popielski and Pick and Spiro on the physiologic action of Witte peptone. The former ascribes the toxicity of the commercial mixture to a substance precipitable by hot absolute alcohol, containing no cholin. This substance he called "vasodilatin." The latter believed the toxic symptoms due to a hypothetical contaminating substance "peptozyme." Underhill[12] after purifying samples of Witte peptone according to the directions of Pick and Spiro, was unable to detect any loss in toxicity. Using purified proteoses obtained by acid hydrolysis of proteins of both animal and vegetable origin he was able to obtain the typical physiologic reactions of proteoses described by Chittenden, Mendel and Henderson. He concludes that "at present there is no occasion for attributing the physiological effects following the injection of proteoses into the circulation to the presence of contaminating substances derived from animal tissue or elsewhere. . . . No method of 'purification' has been found which will deprive proteoses of their characteristic physiological behavior in the circulation; when the chemical make-up of the proteoses is profoundly altered and they lose their chemical identity, the typical physiological action may also be lost."

It seems probable, therefore, that the symptoms of intoxication following intravenous injection of Witte peptone into dogs are due to proteoses contained in the preparation, and is a physiologic reaction differing fundamentally from anaphylaxis in the absence of the phenomenon of sensitization following a suitable incubation period.

The work of Biedl and Kraus stimulated a great deal of investigation and discussion. Richet[13] believes that it is scientifically impossible to compare the physiologic action of peptones with the action of anaphylactic substances. Fall in blood pressure is not sufficient to explain all the symptoms (vomiting, profuse diarrhea, agitation, paraplegia, intoxication, psychic blindness, coma, all phenomena coming on with extreme rapidity) in a dog rendered anaphylactic with actino-congestine. Amyl nitrite, which in small doses produces a fall in blood pressure, does not result in any such grave symptoms. Regarding incoagulability of the blood, he finds that in anaphylaxis produced by mytilo-congestine or actino-congestine, there is no appreciable diminution in the coagulability of the blood. The congestines have the general characters of peptones.

11. Am. Jour. Physiol., 1898-9, 2, p. 142.
12. Ibid., 1903, 9, p. 345.
13. Presse Medicale, 1909, 17, p. [illegible]

Each of the symptoms described by Biedl and Kraus as characteristic of anaphylaxis was studied separately by Salus.[14] He injected intravenously into guinea-pigs a sufficient quantity of magnesium sulphate, sodium citrate or hirudin to produce incoagulability of the blood equal to that in anaphylactic shock without finding any of the lung changes at necropsy. Pure preparations of pepsin were nontoxic for guinea-pigs, only those preparations being toxic which contained albumoses as impurities. The albumoses themselves varied in toxicity. Later he[15] attempted to determine whether the principle in anaphylatoxin is a peptone-like, dialyzable ninhydrin-reacting substance. By dialyzing anaphylatoxin he was never able to obtain a toxic substance in the dialysate. According to this anaphylatoxin does not seem to be a peptone-like substance. Horse serum did not sensitize guinea-pigs to peptone, nor did an injection of peptone render an animal refractory to a second injection of horse serum.

According to Doerr and Muldovan[16] the changes in the lungs in anaphylaxis are not specific for this condition but may be produced by such substances as peptone and saponin. Furthermore, they consider it unsafe to speculate on the identity of the toxin in anaphylaxis and a hypothetical substance in Witte peptone which is supposed to produce the symptoms of "peptone intoxication" until the two have been isolated and identified. Witte peptone is a mixture of substances and the toxic principle contained in it has not yet been identified. They evidently were not familiar with the work of Underhill on proteoses.

DeWaele and Vandevelde[17] observed no symptoms of anaphylaxis following repeated subcutaneous injections of Witte peptone into rabbits. The dosage varied from 0.2-1 gm.

Werbitzky[18] sensitized guinea-pigs with 0.01 gm. horse serum and then injected peptone by various means without producing symptoms except in one animal. The same animals showed the usual reactions of sensitized guinea-pigs when injected with a second dose of horse serum. Peptone did not increase their susceptibility. Doses of peptone which were said to have produced symptoms of anaphylactic shock in dogs (Biedl and Kraus) were found to have absolutely no effect on guinea-pigs. The conclusion is therefore drawn that "peptone intoxication" and protein sensitization are two separate and unrelated phenomena.

The constancy of temperature changes in the guinea-pig during anaphylactic shock is emphasized by Pfeiffer and Mita.[19] There is a fall in temperature often as much as 4 C. They noted a similar drop in temperature after intraperitoneal injection of Witte peptone. By subcutaneous injections of peptone they obtained the Arthus phenomenon or local tissue necrosis at the site of injection. The injection of peptone caused the symptoms to become pronounced in an anaphylactic animal, but peptone was unable to sensitize to itself. The differences between anaphylactic shock and "peptone intoxication" were sufficient to lead them to believe that the two are not identical.

St. Bächer and Wakushima[20] made determinations of the opsonic index in the dog during anaphylactic shock and found that there was a marked drop

[14] Med. Klinik, 1912, 8, p. 1355.
[15] Biochem. Ztschr., 1914, 65, p. 381.
[16] Ztschr. f. Immunitätsforsch., 1910, 7, p. 223.
[17] Biochem. Ztschr., 1910, 30, p. 227.
[18] Compt. rend. de la Soc. de Biol., 1908, 66, p. 23.
[19] Ztschr. f. Immunitätsforsch., 1909, 4, p. 410.
[20] Cent. f. Bakteriol., I, O., 1911, 61, p. 238.

Dogs which received intravenous injections of Witte peptone also showed a marked fall in their opsonic index which varied with the severity of the symptoms.

Friedberger and Mita[21] injected 0.1-0.5 c c of sheep serum intraperitoneally or into the dorsal sac of frogs. One to four weeks later the animals received an intravenous injection of 0.1 c c of the homologus serum. Following the second injection there were characteristic changes in the heart action. Very soon after injection there was a definite slowing of the rate, marked irregularity, and finally the heart stopped in diastole. These changes are attributed to anaphylactic shock, and the authors point out that definite circulatory changes have been observed in anaphylactic shock in warm blooded animals. The effect of various commercial peptones and a sample of pure silk peptone obtained from Abderhalden was then studied. The peptone mixtures had a toxic effect on the isolated frog heart the intensity of which was much less than that of true anaphylactic shock.

Hirschfelder[22] reports that by the intravenous injection of from 5-8 c c of 10% Witte peptone into guinea-pigs he was able to produce the changes in the lungs described by Auer and Lewis as characteristic of anaphylactic shock. This occurred only when the injection was made rapidly. As much as 55 c c intraperitoneally produced no effect.

Manwaring[23] sensitized three dogs against horse serum and after a second injection the animals recovered. They were in no way refractory to Witte peptone but reacted in the usual way. If the mechanism for both reactions were the same we should expect that when animals fail to react against the one they would also fail to react against the other. Manwaring is unwilling to admit that the two phenomena are different, but concludes that the toxic substance formed or set free may be identical in both cases. Loewit[24] repeated the experiments of Manwaring, using rabbits and guinea-pigs. He also found that animals which had been made anti-anaphylactic by recovery from a second injection of horse serum reacted to a single injection of Witte peptone in a typical way. So that in the guinea-pig and rabbit as well as in the dog the exhaustion of one mechanism leaves the other still intact.

Nolf[25] was able to confirm the findings of Biedl and Kraus in dogs, and also believes that anaphylaxis is identical with "propeptone" intoxication.

Calvary[26] found that during anaphylactic shock in the dog there was a lessened flow of lymph which failed to clot. A single injection of protein had no such effect, neither did a mere fall in blood pressure. Witte peptone, on the other hand, had a lymphagogue action. He concludes that if both reactions were the same, their effect on the lymph ought to be the same.

Graetz[27] made an extensive study of the anatomic changes in guinea-pigs dying of anaphylactic shock. He found that the circulatory changes and the changes in the lungs were the same as those following a single injection of Witte peptone.

In a study of the blood changes following intravenous injection of egg-white into dogs, Schittenhelm, Weichardt and Grisshammer[28] found that the

[21] Ztschr. f. Immunitätsforsch., 1911, 10, p. 362.
[22] Jour. Exper. Med., 1910, 12, p. 586.
[23] Ztschr. f. Immunitätsforsch., 1911, 8, p. 589.
[24] Arch. f. Exper. Path. u. Pharm., 1911, 65, p. 337.
[25] Arch. Internat. de Physiol., 1910, 10, p. 37.
[26] München. med. Wchnschr., 1911, 58, p. 670.
[27] Ztschr. f. Immunitätsforsch., 1911, 8, p. 740.
[28] Ztschr. f. Exper. Path. u. Ther., 1912, 10, p. 412.

first injection produced no marked changes. In sensitized animals, following a second injection, there was a marked leukopenia depending on the severity of the anaphylactic reaction. They report similar blood changes following the first injection of large doses of Witte peptone. Silk peptone had no appreciable effect. In their experiments they used as a sensitizing dose 20 c c of egg-white, and repeated this dose at varying intervals. They do not describe the symptoms accepted as anaphylactic, but apparently any animal becoming acutely ill was considered anaphylactic. They also obtained a leukopenia after a third injection of egg white without anaphylaxis resulting, so that leukopenia is not specific for the anaphylactic state.

Salus[29] sensitized guinea-pigs with 0.01 c c of horse serum and found that a nontoxic dose of Witte peptone (1-1.5 c c of a 10% solution) produced no effect. The same animals, however, died of acute anaphylactic shock after receiving an injection of 0.25 c c of horse serum. The horse serum did not sensitize to peptone, nor did an injection of peptone render the animal refractory to a second injection of horse serum. Similar results are reported by Besredka, Ströbel and Jupille.[30] Peptone shock, using Witte peptone, did not in the least protect guinea-pigs sensitized with horse serum against a second injection of horse serum. They also are of the opinion that the mechanism by which the symptoms are produced in "peptone intoxication" is entirely different from that concerned in anaphylactic shock.

Ritz[31] observed that the injection of hypertonic (10%) salt solution into guinea-pigs sensitized with horse serum resulted in milder symptoms of anaphylaxis after the second injection of horse serum. Guinea-pigs received from 1.5-2.2 c c of a 10% Witte peptone solution which was followed by 0.9-1 c c of 30% sodium chlorid solution. The protective effect was not so marked nor as constant as in the case of true anaphylaxis. This author believes that his results furnish additional evidence of a close relationship between anaphylaxis and "peptone intoxication."

Kumagai and Odaira[32] were not able to produce a specific anti-anaphylaxis by the use of Witte peptone. Guinea-pigs were sensitized with sheep serum, and after an incubation period the intoxicating dose was determined. By injecting sub-lethal doses of Witte peptone into sensitized animals they were able to inject twice the usual intoxicating dose of serum without killing the animal. Three times this dose was fatal. Animals which had recovered both from the injection of peptone and from a second injection of sheep serum were given a toxic dose of peptone. The animals showed only a slight protection against "peptone intoxication." The injection of peptone leads only to a slight nonspecific resistance to anaphylaxis, and from the results of their experiments the authors believe that peptone intoxication and anaphylaxis must be considered as separate and distinct phenomena.

In the laboratory of Schittenhelm and Weichardt,[33] an assistant developed severe respiratory symptoms from Witte peptone, and gave a local reaction when it was spread on the skin of his hand. Silk peptone had no such effect. This reaction might easily be accounted for by the presence of a minute amount of histamine.

[29] Biochem. Ztschr., 1914, 65, p. 381.
[30] Ann. de l'Inst. Pasteur, 1913, 27, p. 185.
[31] Ztschr. f. Immunitätsforsch., 1912, 12, p. 644.
[32] Ibid., 1912, 14, p. 391.
[33] Deutsch. med. Wchnschr., 1911, 37, p. 876.

(*b*) *Anaphylaxis with Products of Protein Digestion.*— Rosenau and Anderson[34] found that guinea-pigs sensitized with a mixture of toxin and antitoxic horse serum died when injected 27 days later with antitoxic horse serum to which various ferments had been added and allowed to stand over night at 15 C. The ferments used were takadiastase, pancreatin, rennin, mycrosin, invertin, emulsin, pepsin in acid solution, pepsin in alkaline solution, ingluvin, malt and papain. No attempt was made to determine to what extent, if at all, the proteins had been affected by enzyme action.

Realizing that the discrepancy in results obtained by investigations with Witte peptone might be due to the fact that it is such a heterogeneous mixture of substances, other workers have attempted to study the effects of proteolysis on the anaphylactogenic properties of proteins by using products prepared in the laboratory and purified as nearly as was possible by the use of existing chemical methods.

In his studies of the chemistry of anaphylaxis, Wells[35] found that tryptic digestion of bovine serum until but 8% of its nitrogen remained in coagulable form greatly reduced its sensitizing power. Such a serum sensitized guinea-pigs to normal bovine serum in doses of 0.004 c c but not in doses of 0.0004 c c. Normal bovine serum sensitized to itself in doses of 0.00001 c c. Sensitized guinea-pigs received 5 c c of the digestion mixture intraperitoneally without developing symptoms, showing that it had little or no intoxicating properties. Its effect on animals sensitized to bovine serum was the same. Digestion of serum to this point did not destroy its specificity, in that guinea-pigs sensitized to the digestion mixture did not react to horse serum or milk and were rendered refractory to these substances. After digesting for over 16 months[36] the mixture still contained traces of coagulable material (admixed with the trypsin which had been added?), but gave no biuret reaction. Guinea-pigs receiving doses of 1-5 c c were sensitized so that they reacted slightly but typically, to bovine serum injected 3 weeks later, the most marked reaction occurring in the pigs that had received the 5 c c doses; in no case was the reaction at all severe.

Similar experiments showed that peptic digestion of egg-white destroys its power to intoxicate sensitized guinea-pigs only when practically all coagulable protein has been destroyed. Egg albumin which had been acted on for 26 days by pepsin-HCl until no more coagulable protein was recognizable on heating still was able to sensitize guinea-pigs so that a subsequent injection of egg albumin produced moderate symptoms of anaphylaxis.

Albumoses, peptones, crystallizable amino-acids, etc., obtained by digesting egg white with pepsin and trypsin possessed no power to sensitize or intoxicate guinea-pigs. Some of the products of hydrolysis of coagulated egg albumin possessed a slight power of sensitizing to egg albumin. These experiments indicate that proteins cannot be decomposed much, if any, beyond the coagulable form without losing their anaphylactogenic properties.

Pick and Yamanuchi[37] found that beef serum digested with pepsin-HCl for 15 minutes was still able to sensitize to itself and to undigested beef serum. Rabbits sensitized with the digested serum showed symptoms of anaphylaxis when injected 6 days later with the same mixture and 9 days later when injected with normal serum. The sensitizing and intoxicating

[34] Hyg. Lab., Bull. 36, U. S. Pub. Health Serv., 1907.

[35] Jour. Infect. Dis., 1908, 5, p. 449.

[36] Ibid., 1909, 6, p. 506.

[37] Ztschr. f. Immunitatsforsch., 1909, 1, p. 676.

doses in young rabbits for native serum were not given so that a comparison between the anaphylactogenic properties of the original serum and the digested serum cannot be made. The dosage employed, however, is large so that apparently both the sensitizing and intoxicating properties of beef serum digested with pepsin-HCl for 15 minutes are low. A trypsin-digested mixture, free from coagulable protein and proteoses, yielded results which were not constant. The mixture was not able to sensitize to itself, but in one of a series of animals did sensitize to beef serum. They conclude from their experiments that pure native proteins yield most constant results in anaphylaxis. As the protein content diminishes, fewer positive results are obtained, and the sensitizing and intoxicating doses greatly increased, while the results become less constant.

In their studies of the antigenic properties of the split-products of casein, Gay and Robertson[38] found that a guinea-pig sensitized with 1 c c of 3% casein showed marked symptoms of anaphylactic shock when injected after 23 days with 5. c c of paranuclein. Paranuclein is a product of the partial digestion of casein by pepsin. In a similar manner paranuclein was found to sensitize animals to milk, and to a second injection of paranuclein itself.

A mixture of casein which had been digested with pepsin for 10 days at 36 C., did not sensitize either to a subsequent injection of the same mixture, or to a second injection of paranuclein.

Jobling and Strouse[39] obtained primary and secondary proteoses from Witte peptone by removing all coagulable material and then precipitating with one-half and full saturation of ammonium sulphate. Both fractions were toxic for guinea-pigs and produced death with necropsy findings similar to those in anaphylaxis. Egg white and casein were digested with leukoprotease and the proteoses obtained in the same way, yielded similar results.

Working with proteoses obtained by peptic digestion of beef fibrin prepared by the method of Adler (heteroalbumose and protoalbumose), of Haslam (heteroalbumose, protoalbumose alpha and beta, deuteroalbumose alpha and beta), of Pick (heteroalbumose, protoalbumose, synalbumose and thioalbumose), pepsinfibrinpeptone-beta of Siegfried, and a mixture of abiuret products obtained by the digestion of fibrin with pepsin-trypsin-erepsin, Zunz[40] found that the heteroalbumoses and protoalbumoses were able to sensitize and intoxicate guinea-pigs and rabbits, while synalbumose sensitized only. In animals treated with heteroalbumose, protoalbumose or synalbumose the symptoms of anaphylactic intoxication are not generally so marked as in serum sensitized animals. They usually appear after a definite latent period and are not always marked. In these cases it was necessary to use the lowering of rectal temperature as a criterion of anaphylaxis in guinea-pigs.

If the serum of an animal injected with heteroalbumose or protoalbumose be withdrawn after a suitable interval and incubated with the proteose used for sensitization, a solution is obtained which produces the symptoms of anaphylactic intoxication when injected into a normal animal.

The results of Zunz' experiments clearly show that proteoses are not as effective as serum in the production of anaphylaxis, much larger doses being required to produce less marked symptoms. Specificity is not marked, since an animal sensitized with one proteose will react with any of the other anaphylactogenic proteoses and with beef serum. Species specificity, how-

[38] Jour. Exper. Med., 1912, 16, p. 470.
[39] Ibid., 1913, 18, p. 591.
[40] Ztschr. f. Immunitätsforsch., 1913, 16, p. 580.

ever, is shown, inasmuch as an animal sensitized with proteoses obtained from beef fibrin does not react with horse serum.

Friedberger and Joachimoglu[41] sought to repeat Zunz' work with heteroalbumoses and protoalbumoses. They used preparations furnished them by Zunz. On examining the protocols in the latter's report, they were surprised at the large dose of beef serum used, a dose which in their experience proved toxic to normal animals; 0.15-0.20 c c per 100 gm. body weight was lethal for young guinea-pigs. The lethal dose for animals injected 11 days previously with heteroalbumose or protoalbumose was the same. Such animals showed no symptoms when injected with large doses of horse serum. They therefore attribute the greater susceptibility of guinea-pigs to beef serum as compared with horse serum to the difference in toxicity of the two serums, and not to true sensitization. As compared with normal controls, pigs previously treated with albumose do not show an increased susceptibility to beef serum.

Zunz and György[42] then repeated some of the experiments with proteoses, and again reported that guinea-pigs sensitized with either hetero- or protoalbumose gave anaphylaxis reactions when injected with either or with ox serum, but not with horse serum. The question of the primary toxicity of beef serum as a possible explanation of these results was not discussed.

The sensitizing power of heteroalbumoses seemed to be more marked than their ability to produce anaphylaxis in animals treated with these proteoses. Thus, in such animals the intravenous injection of hetero- or protoalbumose resulted only in mild symptoms of intoxication or even no symptoms whatever. They suggest that perhaps there are varying grades in the sensitizing ability of two heteroalbumose or protoalbumose preparations obtained in exactly the same manner.

A large series of experiments with peptic, tryptic and acid hydrolytic cleavage products of beef and hog muscle is reported by Hailer.[43] In addition, a number of commercial peptone and meat extract preparations were used. The products were tested for coagulable protein, biuret reaction, nitrogen content and precipitability by a homologous precipitin serum. From the results obtained Hailer concludes that it is undoubtedly possible to sensitize guinea-pigs with completely digested protein mixtures (free from coagulable protein, and broken down to the final building stones—the amino-acids—by boiling with sulphuric acid); but that this sensitization is by no means specific since anaphylactic symptoms develop after reinjection of totally unrelated proteins. Despite intensive treatment with a particular kind of protein specific sensitization did not occur when the solution contained large quantities of cleavage products. Similar results were obtained with the commercial products except that reactions were more marked when native protein was present in addition to the cleavage products.

These results are not in agreement with those obtained by Wells[30] who found that tryptic digested serum containing but 8% coagulable nitrogen sensitized to beef serum (40 times the dose of native beef serum required) but did not sensitize to horse serum or milk and were not rendered refractory to these substances. Hailer's conclusions appear to be based on insufficient experimental evidence. Thus, he says that relatively toxic doses were selected because it seemed necessary to flood the animal with large quantities of

[41] Ibid., 1914, 22, p. 522.

[42] Ibid., 1914, 23, 296.

[43] Arb. a. d. k. Gsndhtsamte, 1914, p. 527.

native protein when using substances of relatively weak sensitizing power. As much as 0.6 c c of beef serum was injected intracardially. Friedberger and Joachimoglu[41] found that the lethal dose of beef serum for normal guinea-pigs was 0.15-0.20 gm. per 100 gm. body weight, and while Hailer inactivated his serum, definite symptoms might have resulted from the injection of such doses of beef serum. Since none of his animals died, this remains as a possibility. Suitable controls were not used, as the author depended on the toxic doses of human, horse and swine serums as determined by Uhlenhuth and Händel. No attempt was made to test the intoxicating properties of the digestion mixtures or commercial preparations. The statement that the presence of native protein interferes with the specificity of anaphylaxis by digestion mixtures is contrary to the experience of Wells, whose findings in this respect have already been quoted.

In a series of experiments reported by Schmidt,[44] deuteroalbumose obtained from Witte peptone according to the method of Kutcher was unable to sensitize or intoxicate a sensitized animal.

(*c*) *Vaughan's Protein Poison*.—The work of Vaughan and his students also represents an attempt to prove that anaphylaxis is the result of parenteral digestion of proteins. His theory of anaphylaxis as recently stated in a publication from his laboratory, is that the parenteral introduction of a protein into an animal, the guinea-pig for example, leads to the production of a specific proteolytic ferment by the cells of that animal. Following a suitable incubation period, the injection of a second dose of this same protein, parenterally, results in the liberation of the specific enzyme which then digests the protein molecule with the immediate production of large quantities of poison, sufficient under proper conditions, to kill the animal (Pryer[45]). The experiments of Vaughan on the "protein poison" have served as the basis of this theory.

By extracting egg-white with boiling alcohol (78 C.) containing 2% sodium hydroxid, Vaughan and Wheeler[46] were able to split the protein into a poisonous and nonpoisonous fraction. Earlier experiments (1903) had shown that colon bacillus protein could be thus broken up, and subsequent work in Vaughan's laboratory[47] has shown that all proteins, animal, bacterial and vegetable may be broken up in this manner. When injected into animals, the toxic fraction produces symptoms identical in every particular with those following a second injection of egg white into an animal sensitized against egg white. The minimum lethal dose of the purest preparation of protein poison thus far isolated is 0.0005 gm. (Pryer). Wells[48] found the minimal lethal dose of egg white injected into the circulation of guinea-pigs to be $\frac{1}{10}$ to $\frac{1}{20}$ mg.

Chemical examination of the poisonous product indicated only that the carbohydrate group was absent. For a time Vaughan considered it best to look on the poisonous fraction merely as a cleavage product of whole protein, but still a protein. Recently, however, he has expressed the opinion that "the so-called peptone poison, proteoses and the protein poison are closely related bodies" (Vaughan[49]).

[44] Univ. of Cal. Pub. in Pathology, 1916, 2, p. 157.
[45] Jour. Lab. and Clin. Med., 1916, 1, p. 490.
[46] Jour. Infect. Dis., 1907, 4, p. 476.
[47] Jour. Lab. and Clin. Med., 1916, 1, p. 400.
[48] Jour. Infect. Dis., 1908, 5, p. 449.
[49] Jour. Am. Med. Assn., 1916, 67, p. 1559.

Armit[50] was unable to confirm Vaughan's original experiments. He found that when pure crystallized egg albumin is employed the portion corresponding to the haptophore fraction is unable to sensitize normal guinea-pigs to itself, to the other fraction, or to whole egg albumin. On the other hand, the toxophore fraction is capable of sensitizing to a slight extent. Under certain circumstances it is able to intoxicate when used for a third injection. The hypersusceptibility to pure albumin was not interfered with by the injection of either fraction. On subsequent testing with egg white the guinea-pigs were found to be hypersensitive.

Based on a wide experience in the study of proteoses and "peptone intoxication," Underhill and Hendrix[51] report that Vaughan's crude soluble poison from casein has a more marked physiologic action than any proteose with which they have worked. Its action on blood pressure and blood clotting closely resembles that of the proteoses. Vaughan's preparation was found to be toxic for rabbits in relatively small doses, differing in this respect from the proteoses. By boiling with dilute HCl the toxicity of the protein poison was destroyed, indicating that it is a toxic product of protein hydrolysis.

(*d*) *Precipitins with Derived Proteins.*—In his immunological studies of eel serum, Tchistovitch[52] attempted to produce precipitins against peptone (presumably Witte's). After 5 or 6 injections of 5 c c each of 10% peptone solution into a rabbit, no evidence of a precipitin against peptone could be obtained. The first positive results with the use of Witte peptone as antigen were those of Myers.[53] The commercial product was dissolved in salt solution and the coagulable protein removed by boiling. The cooled filtered solution (concentration not mentioned) was injected intraperitoneally into rabbits. The details of the experiments are not given. Immunization led to the appearance of substances in the serum which produced a precipitum in Witte peptone at 37 C. Control experiments gave absolutely negative results. Heating at 56 C. for half an hour weakened the precipitating power of the serum, but the addition of normal serum restored the original strength. Peptone solution to which normal rabbit serum was added remained perfectly clear. Since the details of these experiments have not been published, a critical discussion is not possible. Later work has not confirmed the results with Witte peptone. Heat inactivation and serum reactivation is not a phenomenon generally observed with precipitins, and this seems to be the only recorded instance in which it occurred.

The statement of Myers that the precipitate formed by the reaction of Witte peptone with a serum immunized against it did not give a biuret reaction, was investigated by Bashford[54] who was able to immunize two goats against Witte peptone, and obtained a large quantity of precipitate by treating the serum with Witte peptone. Comparison of analyses of the precipitated product and the mother substance did not show any striking difference in composition. Bashford points out that proteoses, in the presence of other protein substances in neutral solutions, may yield precipitates which are not the product of a specific immune reaction.

Obermayer and Pick[55] worked with tryptic digested mixtures of pure proteins that did not show the presence of unaltered protein. The mother sub-

[50] Ztschr. f. Immunitätsforsch, 1910, 6, p. 703.
[51] Jour. Biol. Chem., 1915, 22, p. 465.
[52] Ann. de l'Inst. Pasteur, 1899, 13, 406.
[53] Lancet, 1900, 2, p. 98; Cent. f. Bakteriol., 1900, 28, p. 237.
[54] Quoted by Nuttall, Blood Immunity and Relationship, Appendix, Note 2.
[55] Wien. klin. Rund., 1902, 16, p. 277.

stances were egg white, a globulin, conalbumin—the noncrystallizable portion of egg white, and ovomucoid. The injection of trypsin digestion products of these substances into animals led to the rapid appearance of immune products. They believe, therefore, that precipitinogen is not complete protein. The action of pepsin-HCl on proteins which readily produce immune bodies is to destroy their antigenic properties, even while there are considerable quantities of albumoses and peptones present in solution. Witte peptone in their experience had no antigenic properties. It is impossible to give a critical review of this paper by Obermayer and Pick because they give only the results of their experiments and omit a detailed description of the digestion mixtures, the methods used in immunization and the number of experiments on which their results were based. A second paper by the same authors[56] consists of a discussion of the biochemistry of the precipitin reaction with experiments designed to determine on what chemical group in the protein molecule species specificity depends. A biuret-free preparation obtained by long continued autolysis of beef pancreas appeared totally inactive in one experiment. On the other hand, if coagulated beef serum or egg white be subjected to the action of trypsin an immune serum can be obtained with the products of digestion even after the biuret reaction has disappeared. Such an immune serum has a very narrow range of reaction in that it precipitates only the digestion mixture. Its species specificity remains intact, since it does not react with the products of tryptic digestion of horse serum. Immunization of rabbits with products of oxidation of proteins by potassium permanganate in alkaline solution led to the formation of precipitins which were strongly species specific.

After many attempts Michaelis and Oppenheimer[57] were unable to obtain a precipitin by the use of peptic digestion products of proteins which would precipitate the mother substance. They used Riedel peptone, which is obtained through peptic digestion of beef fibrin, Merck egg peptone (peptic digested egg white precipitated with alcohol and dried in vacuo) and pure deutero-albumose obtained from beef by acid hydrolysis. Both commercial preparations probably consisted of mixtures of albumoses. Fifteen animals were used in their experiments. In no instance did the serum of these animals show a specific precipitin. The ability to form a precipitate with a specific immune serum was lost even when considerable heat coagulable material was present in the digestion mixture.

In the case of tryptic digestion, as long as coagulable protein was demonstrable in the solution, it was precipitable by precipitin. After digestion for several weeks with large quantities of trypsin, until protein had entirely disappeared, the precipitability of the mixture by precipitin was completely lost, and it was no longer possible to obtain a precipitin for the mother protein by injecting this substance.

Rostoski and Sacchonagi[58] worked with pepsin and trypsin digestion products of horse serum albumin. They were not able to get rid of the last traces of coagulable protein in the tryptic digestion. Albumoses were obtained by half and full saturation with ammonium sulphate. The filtrates contained peptones. The serum of injected animals yielded a definite precipitate when added to the solutions used for injection. Very marked results were obtained with the ring test of Ascoli, in which serum and solution were placed in narrow

56 Wien. klin. Wchnschr., 1906, 19, p. 327.
57 Arch. f. Physiol., 1902, Supplement, p. 343.
58 Ztschr. f. klin. Med., 1903-04, 51, p. 187.

test tubes. After several minutes a heavy ring-shaped precipitate formed, almost diffuse, finally falling to the bottom. Numerous control experiments using normal serum against the various substances were always negative. The precipitins in this case were not specific, in that the serum of an animal immunized against one of the solutions reacted with all of them. Rostoski and Sacchonagi conclude from their experiments that precipitins can be produced against the products of gastric and pancreatic digestion, even the peptones. They ascribe the failure of previous workers to the use of related proteins.

Michaelis[59] injected a preparation of beef serum partially digested with pepsin-HCl, intraperitoneally into a rabbit at 4-day intervals. Six days after the third injection the serum of the animal was tested. It showed an active precipitin for the digested beef serum producing a marked opalescence almost as soon as the mixture was added. This rapidly turned to a precipitate and settled out. The reaction did not occur in as high dilution as native serum precipitin. With small amounts of normal horse serum a precipitate was formed which dissolved on the addition of an excess of serum. When tested with pseudoglobulin there was no reaction; it reacted well, however, with euglobulin and albumin. If enough serum was added to redissolve the precipitate first formed, the addition of a drop of digestion mixture produced an opalescence. The precipitin against partially digested serum did not react with a digestion mixture from which all coagulable material had been removed either by prolonged digestion or by heating.

Pozerski and Pozerska[60] were unable to demonstrate the presence of specific precipitins in the serum of a dog immunized against Witte "peptone,"

Levene[61] worked with proto- and deuteroalbumoses obtained from Witte "peptone" by precipitation with one-half and full saturation with ammonium sulphate. The serum of rabbits injected with either proteose formed a precipitate with both antigens. Relatively large quantities of serum (as much as 0.4 c c) and antigen (2% solution) were used in the tests. Schmidt[62] used deuteroalbumose from Witte "peptone" and did not obtain a precipitin reaction with the antiserum.

Recently Lampé[63] has reported positive results with various peptones. These were obtained from the crystalline lens, brain, placenta, thyroid, thymus, lung, silk and gliadin. The smallest quantities used activated hemolysis. Normal serum was found to contain no antibodies to these peptones, but antibodies to some may occur in human serum in disease or in rabbits after immunization. Specificity was not absolute in that following protein injections antibodies to the corresponding peptones appeared. Unfortunately I have been unable to gain access to the original article and am thus unable to determine just what tests for antibody production were employed, but the ability of a substance to activate hemolysis is not a sufficient test of its antigenic properties.

(e) Complement Fixation with Protein Derived Products.—Friedberger and Gay and Robertson using the substances mentioned under the discussion of the anaphylaxis reaction were unable to demonstrate the presence of an antibody to any of the substances except whole protein. Schmidt also reports negative results with his deuteroalbumose.

[59] Deutsch. med. Wchnschr., 1902, 28, p. 733.

[60] Compt. Rend. Soc. Biol., 1911, 70, p. 592.

[61] Jour. Med. Research, 1904, 12, p. 195.

[62] Univ. of Cal. Pub. in Pathology, 1916, 2, p. 157.

[63] Deutsch. Arch. f. klin. Med., 1916, 119, p. 113. Physiological Abstracts, 1916, i, p. 224.

By digesting proteins (horse serum) with an alcoholic solution of sulphuric acid (48 hours at 62-63 C.), Landsteiner and Prasek[64] were able to produce an immune serum in rabbits which reacted with similarly modified serum proteins of various animals (beef, chicken, rabbit) and even with edestin but not with unaltered horse serum.

Landsteiner and Jablons[65] report that rabbit serum treated with alcoholic sulphuric acid produces complement binding antibodies when injected into rabbits.

SUMMARY OF LITERATURE

The bulk of evidence indicates that "peptone shock" is not an anaphylactic phenomenon. Other substances totally unrelated to anaphylactogens, such as saponins and hirudin may give similar symptoms. The work of Underhill and others has shown quite conclusively that the proteoses contained in the various "peptone" preparations are responsible for their physiologic action. "Peptone shock" differs fundamentally from anaphylaxis in the absence of the phenomenon of sensitization following a suitable incubation period, and in the relatively large quantity of "peptone" required to produce a reaction. Experiments with products of protein digestion have shown that proteins cannot be disintegrated much if any beyond the coagulable form without losing their sensitizing and intoxicating properties. Positive experiments reported with proteoses as anaphylactogens have not been fully confirmed.

The single recorded instance of precipitin formation against Witte peptone has never been confirmed, all subsequent work showing that it has no antigenic properties.

Most of the precipitin experiments with protein digestion products were undertaken to determine on what group in the protein molecule specificity depends, rather than to test the effect of disintegration on their antigenic properties. The entire literature on precipitins is in a chaotic state, the great bulk of it consisting of theoretical discussions. Instead of testing against the digestion mixtures, the serums obtained by their use have often been tested against the mother substance alone. Separating the wheat from the chaff, what little remains seems to parallel the results with the anaphylaxis reaction. Precipitinogen seems to be more resistant to tryptic digestion than to peptic digestion, corresponding to proteins in general in this respect.

Experiments with the complement fixation reaction have been negative.

64 Ztschr. f. Immunitätsforsch., 1913, 20, p. 211.

65 Ibid., 1914, 20, p. 618.

We might mention at this point that the evidence for the proteose nature of toxins is doubtful, and that Whipple[65a] considers intestinal intoxication due to proteoses.

THE CHEMISTRY OF PROTEOSES

The proteoses are not a chemically definable group of substances, inasmuch as their exact chemical composition is practically unknown. Our knowledge of proteoses has resulted largely from attempts to determine the structure and composition of the protein molecule through methods of analysis. By hydrolytic cleavage, proteins are decomposed, yielding products of lower molecular weight. Among the first of these are the proteoses regardless of the agent employed for hydrolysis, whether by the action of enzymes, dilute acids or superheated steam. So that proteoses, or albumoses, as they are often called have been defined as a group of derived proteins. Analysis has shown that they differ but little in their fundamental composition from the mother proteins.

Albumoses are identified almost wholly by their physical properties and physiologic action. That they consist of smaller molecules than the proteins from which they are derived is evidenced by the fact that they are somewhat more diffusible. Their separation and classification have been largely worked out by Kühne and his pupils, and is based on the fact that they are precipitated by solutions of neutral salts of different strengths.

Kühne and Chittenden[66] divide the primary albumoses into proto- and hetero-albumose. During peptic digestion these give rise to the secondary albumoses or deutero-albumoses. Differences in solubility and precipitability by sodium chlorid led to the separation of four different albumoses. Kühne's[67] method of separating albumoses from peptones consists in saturating with ammonium sulphate while hot, a solution containing a mixture of the two of neutral, alkaline and acid reactions. Although apparently simple, their method does not always succeed, and Kühne and Chittenden have found that the filtrates which are supposed to contain only peptones sometimes contain definite quantities of proteoses. The difficulty seems to be in the adjustment of the reaction at the different stages of precipitation.

Proteoses thus obtained were found to be water-soluble; with sodium chlorid and nitric acid they formed precipitates in the cold which dissolved on heating, and they all gave the biuret reaction.

The work of Kühne and Chittenden was carried a step farther by Neumeister[68] who perfected a method of separating deuteroalbumoses from a

[65a] Jour. Am. Med. Assn., 1916, 67, p. 15.
[66] Ztschr. f. Biol., 1884, 20, p. 11.
[67] Ibid., 1892, 29, p. 1.
[68] Ibid., 1887, 23, p. 381.

mixture of albumoses. Neumeister's method is based on the conception that, in the process of protein cleavage, albumoses are formed in two stages. The first stage results in the formation of proto- and hetero-albumoses, which he has termed primary albumoses; in the second stage each of the primary albumoses yields a deuteroalbumose, or secondary albumose.

E. P. Pick[69] working with Witte peptone was able to isolate four fractions by precipitate with ammonium sulphate of varying degrees of saturation.

The method of fractional precipitation of proteoses has been severely criticized by Haslam[70] who called attention to the fact that there are no chemical tests by which we are able to prove that the various fractions are not mixtures, and that reprecipitation does not serve to remove all traces of impurities, especially other albumoses. In order to avoid this difficulty, Haslam suggests that where possible, the filtrate should be tested for the substance which it is desired to remove by precipitation. Where no such test exists, the amount of organic nitrogen should be determined by the Kjeldahl method in the original filtrate after resolution and reprecipitation. The substance should not be considered pure until the organic nitrogen in the filtrate becomes constant. Washing the precipitate was found to be of no value because the impurities are intimately admixed with the albumoses and washing affects only the surface particles.

PRELIMINARY EXPERIMENTS WITH WITTE "PEPTONE" PRODUCTS

It seemed desirable to test the antigenic properties of Witte "peptone," in the first place to get an idea of the activity of proteoses in this respect, and in the second place to determine if possible why the results of other workers are so conflicting. Three preparations were obtained as follows:

1. Hot alcohol soluble proteose prepared according to the method of Gibson[71] who proved by its physiologic activity on dogs that it was a true proteose. Witte "peptone," 150 gm., were boiled several hours with 1 liter of 80% alcohol under a reflux condenser. The alcoholic solution was filtered through a hot water funnel and the proteose precipitated in a semi-crystalline form in a freezing mixture. The extraction was repeated several times and the fractions combined. The final product was pulverized in a mortar, yielding a brownish-white powder readily soluble in water. From a watery solution it could be precipitated by ⅔ saturation with ammonium sulphate and the filtrate gave only an opalescence with an excess of ammonium sulphate. The filtrate from ¾ saturation gave no further precipitate on the addition of saturated ammonium sulphate. This preparation was used as antigen 1.

2. Cold alcohol soluble material prepared by evaporating to dryness the filtrate from the above and pulverizing in a mortar. This was a light yellow powder, readily soluble in water, and was used as antigen 2.

3. The residue from the alcohol extraction was dissolved as much as possible in hot water, evaporated to a syrupy consistence, and precipitated with 3 times its volume of 95% alcohol, yielding a sticky mass. This was washed with absolute alcohol, followed by ether and dried in a desiccator. The dried material was powdered as above and used as antigen 3.

4. Beef serum was used as control antigen 4.

[69] Ztschr. f. Physiol. Chem., 1898, 24, p. 246.

[70] Jour. Physiol., 1905, 32, p. 267; 1907, 36, p. 164.

[71] Philippine Jour. Science, 1914, 9B, p. 499.

ANIMAL EXPERIMENTS

The first series of animals were immunized by the intensive method. Intraperitoneal injections were given on three successive days of 0.5, 1.0 and 1.5 gm., respectively, and the animals bled on the 10th day after the first injection. The serums were then tested for precipitins and complement binding substances. For the precipitin reaction a 1% antigen solution was used in dilutions varying from 0.5-0.0009 c c with the usual controls. In testing out each serum all the antigens were used to determine nonspecific reactions. For the complement fixation reactions a 1% solution of the antigens were used in dilutions ranging from $\frac{1}{20}$ to $\frac{1}{10{,}240}$ c c with suitable controls. In this case also all the antigens were tested against each serum. The serums of animals injected with antigens 1, 2 and 3 failed to show the presence of antibodies for any of the antigens used. Animals treated in the same way, using 5, 10 and 15 c c of beef serum showed in the case of precipitins a positive reaction down to the 5th tube, and complete inhibition in the first 4 tubes with partial inhibition in the rest. With the other preparations as antigens, using the same system, there were no reactions.

In order to be certain that the negative results were not due to insufficient treatment of animals, another series of rabbits were injected with increasing doses of antigen at 3-day intervals, beginning with 0.5 gm., and increasing by 0.1 gm. at each subsequent injection. These animals were injected over a period of one month. The results were the same, and no evidence of antibody formation could be demonstrated. Therefore, the three fractions prepared from Witte's "peptone" possessed no power to stimulate the production of precipitins or complement binding antibodies, either for themselves or for beef serum — the mother substance. Neither did they react with the serum of rabbits immunized against beef serum.

Anaphylaxis experiments showed that the Witte "peptone" antigens possessed only very slight anaphylactogenic properties. Guinea-pigs received intraperitoneal injections of 25 mg. of the substance to be tested and 3 weeks later a second injection of 50 mg. In only one or two cases did the animals become sick. In no case did such severe reactions result as follow on the re-injection of beef serum into an animal sensitized to beef serum. For the most part the reactions consisted of vigorous scratching 15 minutes after reinjection. Guinea-pigs sensitized with Witte "peptone" products did not react to beef

serum, nor was the reverse combination any more effective. Preliminary experiments with normal guinea-pigs showed that the preparations in the dosage employed were nontoxic. The protocols are contained in table 1.

TABLE 1

ANAPHYLAXIS EXPERIMENTS WITH PRODUCTS FROM WITTE'S "PEPTONE"

Sensitizing Dose (Intraperitoneally)	Days Interval	Second Injection	Results
1. 0.025 gm. Antigen 1	19	0.050 gm. Antigen 1	Slight roughing, scratching
2. 0.025 gm. Antigen 1	19	0.050 gm. Antigen 1	Scratching
3. 0.025 gm. Antigen 1	19	0.050 gm. Antigen 1	Scratching
4. 0.025 gm. Antigen 1	19	0.050 gm. Antigen 1	No symptoms
5. 0.025 gm. Antigen 2	19	0.050 gm. Antigen 2	No symptoms
6. 0.025 gm. Antigen 2	19	0.050 gm. Antigen 2	No symptoms
7. 0.025 gm. Antigen 2	19	0.050 gm. Antigen 2	Scratching
8. 0.025 gm. Antigen 2	19	0.050 gm. Antigen 2	No symptoms
9. 0.025 gm. Antigen 3	19	0.050 gm. Antigen 3	Roughened hair, scratching
10. 0.025 gm. Antigen 3	19	0.050 gm. Antigen 3	Scratching
11. 0.025 gm. Antigen 3	19	0.050 gm. Antigen 3	Slightly upset, rapid resp.
12. 0.025 gm. Antigen 3	19	0.050 gm. Antigen 3	Scratching
13. 0.025 gm. Antigen 1	19	0.1 c c heated beef serum	No reaction
14. 0.025 gm. Antigen 2	19	0.1 c c heated beef serum	Scratching
15. 0.025 gm. Antigen 3	19	0.1 c c heated beef serum	No reaction
16. 0.1 c c heated beef serum	21	0.1 c c heated beef serum	Typical shock, recovery
17. 0.1 c c heated beef serum	21	0.050 gm. Antigen 1	No reaction
18. 0.1 c c heated beef serum	21	0.050 gm. Antigen 2	No reaction
19. 0.1 c c heated beef serum	21	0.050 gm. Antigen 3	Scratching, late paralysis, not specific
20. 0.1 c c heated beef serum	21	0.050 gm. Antigen 3	No reaction
21. 0.1 c c heated beef serum	21	0.050 gm. Antigen 3	No reaction

Beef serum does not sensitize to products of Witte "peptone," nor does it produce "shock" in animals sensitized to such products.

Witte "peptone" preparations, therefore, possess a slight power of sensitizing to themselves. They are not able to sensitize to beef serum, nor do they produce symptoms of anaphylactic intoxication in guinea-pigs sensitized to beef serum.

EXPERIMENTS WITH PROTEOSES FROM EGG WHITE

(*a*) *Method of Preparation.*—In order to avoid the possibility of introducing protein substances which might be difficult to get rid of in a digestion mixture, enzymes were not used. The method employed was essentially that of Chittenden, Mendel and Henderson.[72] The whites of 6 dozen eggs were coagulated by pouring slowly into a large volume of boiling water to which enough acetic acid had been added to make it distinctly acid to litmus paper. The coagulum was collected on a clean towel, the water squeezed out as much as possible and ground through a fine-meshed copper sieve, in order to obtain the maximum surface for hydrolytic action. The coagulated egg white in a finely divided state, was suspended in an equal volume of water and autoclaved for 10 hours with steam under 10 lbs. pressure. The mixture was then filtered and the filtrate slightly acidified with acetic acid to precipitate any coagulable protein present, again filtered, and the filtrate rendered slightly alkaline with ammonium hydroxid. The neutralization precipitate was filtered

[72] Am. Jour. Physiol., 1898-99, 12, p. 142.

off, and the solution boiled on the water-bath to drive off any excess ammonia, and finally concentrated to a small volume. This final product was filtered until clear. To the filtrate was then added enough cold saturated $(NH_4)_2SO_4$ solution to make it 1/4 saturated, that is, to every 300 c c of filtrate 100 c c of saturated sulphate solution was added. The brownish sticky precipitate was filtered off and allowed to dry on the filter paper. In the same way products were obtained by adding to the filtrate enough saturated $(NH_4)_2SO_4$ solution to yield 1/3, 1/2, 2/3, 3/4 and full saturated fractions, that is, to the filtrate of the 1/4 saturated fraction was added the calculated volume necessary to bring the concentration to 1/3 saturation, etc. The insoluble residue from the original filtrate was again suspended in water and again autoclaved. When the yield in proteoses became small a final hydrolysis with 0.8% HCl was attempted and the residue brought into solution. In order to obtain enough material for immunizing animals it was necessary to repeat the process many times. The yield of proteoses was inconstant at different times, in some instances being abundant, while in others quite disappointing. This has been the experience of most workers with proteoses, and makes the task of obtaining proteoses by hydrolysis tedious and time-consuming. Obviously we are dealing here with a chemical reaction which cannot be controlled. At one time the process may be interrupted at a point when the maximum yield of proteoses results, at another time when the disintegration of the molecule has gone beyond this stage. In the case of a compound of the complex structure of the protein molecule there is no way of determining the stage of optimum production of proteoses. Where the conditions of chemical equilibrium are changing as constantly as in the hydrolysis of proteins, the time when the process should be interrupted is largely a matter of trial and error.

The preparations were purified by dissolving the dried material on the filter papers, discarding the insoluble portions, and determining the amount of ammonium sulphate present by the Folin aeration method.[73] For this purpose 10 c c of the solution to be tested was placed in the receiving cylinder of the Folin apparatus. This was then covered with 5 c c paraffin oil, and 5 gm. dry Na_2CO_3 dusted over the layer of oil. The cylinder was stoppered quickly, and by means of a current of air the liberated ammonia was passed into another cylinder containing 20 c c of N/20 H_2SO_4. Vigorous aeration was continued for 1½ hours, after which the acid solution was titrated with N/10 NaOH using congo red as indicator. From the amount of ammonia thus determined, the concentration of $(NH_4)_2SO_4$ in the total volume of solution was calculated and sufficient saturated $(NH_4)_2SO_4$ solution added to bring it to the desired concentration (1/4 saturation, 1/3 saturation, etc., in the corresponding fractions). The filtrate was added to the succeeding fraction. The precipitates were washed with the corresponding saturation of $(NH_4)_2SO_4$ solution and the whole process repeated. The final products were obtained by precipitation with 3 volumes of alcohol, washing with a small amount of ether, and drying in a desiccator. The result in all cases was a white amorphous powder readily soluble in water. The yield was as follows: 1/4 saturated fraction, 0.7 gm.; 1/3 saturated fraction, 4.2 gm.; 1/2 saturated fraction, 11 gm.; 2/3 saturated fraction, 25 gm.; 3/4 saturated fraction, 12 gm.; 4/4 saturated fraction, 49 gm.

[73] Mathews, Physiological Chemistry, 1916, p. 961.

Sufficient material for testing the physiologic action of the preparations was obtained only with the ½, ⅔, ¾ and ¼ saturated products. These were injected intravenously into dogs in concentrations of 0.06 gm. per kg. of body weight and showed in all cases the typical drop in blood pressure characteristic of all proteoses.

The protocols of our immunization experiments show that the small amounts of ammonium sulphate contained in the preparations did not interfere with the biologic reactions.

(*b*) *Animal Experiments.*—Rabbits were used to test for the production of precipitins and complement binding antibodies. In the first series of experiments, rabbits were injected in pairs with each fraction as follows: 0.1 gm. dissolved in 5 c c of water was injected intravenously on each of 3 successive days, the animals allowed to rest 3 days, after which 3 injections of 0.1 gm. each were again given on successive days. Each animal thus received 0.6 gm. of the preparation to be tested. A second series of rabbits was injected intravenously with proteose preparations, a pair of animals for each fraction, starting with 0.1 gm. and increasing by 0.1 gm. at each succeeding injection, space at 3-day intervals over a period of 1 month. The rabbits were bled and their serums tested for antibodies during the course of treatment, and the final injection consisted of an intraperitoneal injection of 0.5 gm., the deciding test being made with serum withdrawn on the 10th day after the last injection. As controls, rabbits were treated with filtered fresh egg white solution, in exactly the same way using 0.1 c c egg white for each 0.1 gm. proteose. Owing to the small yield of proteoses of 1/4 saturation with ammonium sulphate, only a limited number of experiments were possible with this fraction.

The technic in testing for precipitins and complement binding antibodies was the same as described in connection with the experiments on Witte "peptone" products. The serums of aniamls injected with 1/4, 1/3, 1/2 and 2/3 saturation products showed no evidence of production of either precipitins or complement binding antibodies.

The results of the experiments show that the ¾ and ¼ saturated products were not as efficient antibody producers as egg white, but that they possess definite antigenic power for both precipitin and complement binding antibodies. In the complement fixation experiments the possible anticomplementary effect of ammonium sulphate contained in the preparations was carefully controlled. In treating rabbits for antigen production we found that two of our animals died suddenly following the third intravenous injection, with typical symptoms of anaphylactic shock. Such reactions never occurred with any of the other fractions, and furnish additional evidence that the ¾ and ¼ saturated fractions possess antigenic properties not possessed by the others.

The following are typical protocols of experiments with 3/4 and 4/4 saturation products and egg white as control.

TABLE 2

COMPLEMENT FIXATION TESTS.* EGG-WHITE IMMUNE RABBIT SERUM

Antigen† Dilution 1% Solution	1/4 Saturated	1/3 Saturated	1/2 Saturated	2-3 Saturated	3/4 Saturated	4/4 Saturated	Egg-white
1. 1:20	Hemolysis complete	Hemolysis complete	Hemolysis complete	Hemolysis complete	++	++	++++
2. 1:40	Hemolysis complete	Hemolysis complete	Hemolysis complete	Hemolysis complete	+	Slight fixation	++++
3. 1:80	Hemolysis complete	Hemolysis complete	Hemolysis complete.	Hemolysis complete	+	Slight fixation	++++
4. 1:160	Hemolysis complete	Hemolysis complete	Hemolysis complete	Hemolysis complete	+	Slight fixation	++++
5. 1:320	Hemolysis complete	Hemolysis complete	Hemolysis complete	Hemolysis complete	+	Slight fixation	++++
6. 1:640	Hemolysis complete	Hemolysis complete	Hemolysis complete	Hemolysis complete	+	Slight fixation	++
7. 1:1,280	Hemolysis complete	Hemolysis complete	Hemolysis complete	Hemolysis complete	Slight fixation	Slight fixation	+
8. 1:2,560	Hemolysis complete	Hemolysis complete	Hemolysis complete	Hemolysis complete	Slight fixation	Slight fixation	+
9. 1:5,120	Hemolysis complete	Hemolysis complete	Hemolysis complete	Hemolysis complete	Slight fixation	Slight fixation	+
10. 1:10,240	Hemolysis complete	Hemolysis complete	Hemolysis complete	Hemolysis complete	Hemolysis complete	Hemolysis complete	Hemolysis complete
11. Normal salt	Hemolysis complete	Hemolysis complete	Hemolysis complete	Hemolysis complete	Hemolysis complete	Hemolysis complete	Hemolysis complete
12. 1:20 + normal rabbit serum (56 C.)	Hemolysis complete	Hemolysis complete	Hemolysis complete	Hemolysis complete	Hemolysis complete	Hemolysis complete	Hemolysis complete
13. 1:20 + normal salt	Hemolysis complete	Hemolysis complete	Hemolysis complete	Hemolysis complete	Hemolysis complete	Hemolysis complete	Hemolysis complete

* The hemolytic system employed was: Amboceptor—rabbit serum immune to sheep's corpuscles, 2 units. Complement fresh guinea-pig serum, titrated before use, 1.2 units. Sheep's corpuscles, thoroughly washed fresh, 2.5% suspension. Anticomplementary dose of antigen determined and 1/4 anticomplementary dose used.

† 0.1 c c antiserum (56 C. ½ hr.) in each of first 10 tubes, normal salt sol. q. s. 4 c c in all tubes.

TABLE 3

PRECIPITIN TEST. EGG-WHITE IMMUNE RABBIT SERUM. 0.1 C C ANTISERUM IN EACH OF FIRST 10 TUBES, NORMAL SALT SOLUTION Q. S. 2 C C IN ALL TUBES

Dilution of Antigen 1% Solution C C	Antigens						
	1/4 Saturated	1/3 Saturated	1/2 Saturated	2/3 Saturated	3/4 Saturated	4/4 Saturated	Egg-white Saturated
1. 0.5	—	—	—	—	slight	slight	[illegible]
2. 0.25	—	—	—	—	slight	slight	[illegible]
3. 0.125	—	—	—	—	slight	slight	[illegible]
4. 0.0625	—	—	—	—	slight	slight	[illegible]
5. 0.03125	—	—	—	—	—	—	+
6. 0.0156	—	—	—	—	—	—	+
7. 0.0078	—	—	—	—	—	—	+
8. 0.0039	—	—	—	—	—	—	[illegible]
9. 0.0019	—	—	—	—	—	—	+
10. 0.0009	—	—	—	—	—	—	+
11. no antigen	—	—	—	—	—	—	—
12. 0.5 normal rabbit serum	—	—	—	—	—	—	—
13. 0.5 normal salt	—	—	—	—	—	—	—

TABLE 4

PRECIPITIN TEST: ¾ SAT. RABBIT IMMUNE SERUM. 0.1 C C ANTISERUM IN EACH OF FIRST 10 MIXTURES, NORMAL SALT SOLUTION Q. S. 2 C C IN ALL TUBES

	Dilution of Antigen 1% Solution, C C	3/4 Saturation as Antigen	Egg white as Antigen
1.	0.5	+	+
2.	0.25	+	+
3.	0.125	+	+
4.	0.0625	+	+
5.	0.03125	+	+
6.	0.0156	+	+
7.	0.0078	Faint trace	+
8.	0.0039	Faint trace	+
9.	0.0019	Faint trace	No precipitation
10.	0.0009	Faint trace	No precipitation
11.	no antigen	No precipitation	No precipitation
12.	0.5 + normal rabbit serum	No precipitation	No precipitation
13.	0.5 + normal salt	No precipitation	No precipitation

TABLE 5

PRECIPITIN TEST: 4/4 SAT.-RABBIT-IMMUNE SERUM. 0.1 C C ANTISERUM IN EACH OF FIRST 10 MIXTURES, NORMAL SALT SOLUTION Q. S. 2 C C IN ALL TUBES

	Dilution of Antigen 1% Solution, C C	4/4 Saturation as Antigen	Egg white as Antigen
1.	0.5	+++	++
2.	0.25	++	—
3.	0.125	+	—
4.	0.0625	—	—
5.	0.03125	—	—
6.	0.0156	—	—
7.	0.0078	—	—
8.	0.0039	—	—
9.	0.0019	—	—
10.	0.0009	—	—
11.	no antigen	—	—
12.	0.5 + normal rabbit serum	—	—
13.	0.5 + normal salt	—	—

TABLE 6

COMPLEMENT FIXATION TESTS: ¾ SAT.-RABBIT-IMMUNE SERUM, 0.1 C C ANTISERUM (56 C., ½ HOUR) IN EACH OF FIRST 10 MIXTURES, NORMAL SALT SOLUTION Q. S. 4 C C IN ALL TUBES

	Antigen Dilution 1% Solution	3/4 Saturation as Antigen	Egg white as Antigen
1.	1:20	+++	+++
2.	1:40	+++	+++
3.	1:80	+++	+++
4.	1:160	+++	+++
5.	1:320	+++	+++
6.	1:640	+++	+++
7.	1:1,280	+++	+++
8.	1:2,560	+++	+++
9.	1:5.120	+++	+++
10.	1:10,240	Hemolysis complete	Hemolysis complete
11.	Normal NaCl	Hemolysis complete	Hemolysis complete
12.	1:20 + 0.1 normal rabbit serum	Hemolysis complete	Hemolysis complete
13.	1:20 + normal NaCl	Hemolysis complete	Hemolysis complete

TABLE 7

COMPLEMENT FIXATION TESTS: 4/4 SAT.-RABBIT-IMMUNE-SERUM, 0.1 C C ANTISERUM (56 C., 1/2 HOUR) IN EACH OF FIRST 10 MIXTURES, NORMAL SALT SOLUTION Q. S. 4 C C IN ALL TUBES

	Antigen Dilution 1% Solution	4/4 Saturation as Antigen	Egg white as Antigen
1.	1:20	++++	++
2.	1:40	++	Hemolysis complete
3.	1:80	+	Hemolysis complete
4.	1:160	Slight inhibition	Hemolysis complete
5.	1:320	Slight inhibition	Hemolysis complete
6.	1:640	Slight inhibition	Hemolysis complete
7.	1:1,280	Slight inhibition	Hemolysis complete
8.	1:2,560	Slight inhibition	Hemolysis complete
9.	1:5,120	Hemolysis complete	Hemolysis complete
10.	1:10,240	Hemolysis complete	Hemolysis complete
11.	Normal NaCl	Hemolysis complete	Hemolysis complete
12.	1:20 + 0.1 normal rabbit serum	Hemolysis complete	Hemolysis complete
13.	1:20 + normal NaCl	Hemolysis complete	Hemolysis complete

TABLE 8

ANAPHYLAXIS EXPERIMENTS WITH PROTEOSES FROM EGG WHITE[4]

	Sensitizing Dose	Days Interval	Second Injection	Results	Subsequent Injections
1.	0.010 gm. 1/4 sat.	21	0.025 gm. 1/4 sat.	No reaction	4 days later, 1 c c 50% egg white, no reaction
2.	0.010 gm. 1/4 sat.	21	0.025 gm. 1/4 sat.	No reaction	4 days later, 1 c c 50% egg white, no reaction
3.	0.010 gm. 1/4 sat.	21	0.025 gm. 1/4 sat.	No reaction	4 days later, 1 c c 50% egg white, no reaction
4.	0.010 gm. 1/3 sat.	21	0.025 gm. 1/3 sat.	No reaction	4 days later, 1 c c 50% egg white, sick in 15 min., recovered in 30 minutes
5.	0.010 gm. 1/3 sat.	21	0.025 gm. 1/3 sat.	No reaction	
6.	0.010 gm. 1/3 sat.	21	0.025 gm. 1/3 sat.	No reaction	
7.	0.010 gm. 1/2 sat.	21	0.025 gm. 1/2 sat.	No reaction	1 c c 50% egg white, no reaction
8.	0.010 gm. 1/2 sat.	21	0.025 gm. 1/2 sat.	No reaction	1 c c 50% egg white, no reaction
9.	0.010 gm. 1/2 sat.	21	0.025 gm. 1/2 sat.	No reaction	1 c c 50% egg white, no reaction
10.	0.010 gm. 1/2 sat.	21	0.025 gm. 1/2 sat.	No reaction	1 c c 50% egg white, no reaction
11.	0.010 gm. 2/3 sat.	21	0.025 gm. 2/3 sat.	No reaction	1 c c 50% egg white, no reaction
12.	0.010 gm. 2/3 sat.	21	0.025 gm. 2/3 sat.	No reaction	1 c c 50% egg white, no reaction
13.	0.010 gm. 2/3 sat.	21	0.025 gm. 2/3 sat.	No reaction	1 c c 50% egg white, no reaction
14.	0.010 gm. 2/3 sat.	21	0.025 gm. 2/3 sat.	No reaction	1 c c 50% egg white, no reaction
15.	0.010 gm. 3/4 sat.	23	0.025 gm. 3/4 sat.	Vigorous scratching	1 c c 50% egg white, vigorous scratching
16.	0.010 gm. 3/4 sat.	23	0.025 gm. 3/4 sat.	Vigorous scratching, roughing of fur	1 c c 50% egg white, vigorous scratching
17.	0.010 gm. 3/4 sat.	23	2 c c 10% egg white	Vigorous scratching	1 c c 50% egg white, heaving respiration
18.	0.010 gm. 3/4 sat.	23	2 c c 10% egg white	Scratching	

TABLE 8—*Continued*

ANAPHYLAXIS EXPERIMENTS WITH PROTEOSES FROM EGG WHITE*

Sesitizing Dose	Days Interval	Second Injection	Results	Subsequent Injections
19. 0.010 gm. 4/4 sat.	23	0.025 gm. 4/4 sat.	Immediate convulsive breathing, recovery, 5 minutes	
20. 0.010 gm. 4/4 sat.	23	0.025 gm. 4/4 sat.	Roughing of fur, slight difficulty in breathing	
21. 0.010 gm. 4/4 sat.	23	2 c c 10% egg white	Scratching	1 c c 50% egg white, vigorous scratching
22. 0.010 gm. 4/4 sat.	23	2 c c 10% egg white	Scratching	1 c c 50% egg white, vigorous scratching
23. 2 c c 5% egg white	21	0.050 gm. 3/4 sat.	No reaction	1 c c 50% egg white, heaving resp.
24. 2 c c 5% egg white	21	0.050 gm. 3/4 sat.	No reaction	1 c c 50% egg white, vigorous scratching
25. 2 c c 5% egg white	21	0.050 gm. 4/4 sat.	No reaction	1 c c 50% egg white, vigorous scratching
26. 2 c c 5% egg white	21	0.050 gm. 4/4 sat.	No reaction	1 c c 50% egg white, no reaction
27. 2 c c 5% egg white	21	0.050 gm. 3/4 sat.	Vigorous scratching	1 c c 50% egg white, no reaction
28. 2 c c 5% egg white	21	0.050 gm. 3/4 sat.	Vigorous scratching	4 days later, 1 c c egg white, no reaction
29. 2 c c 5% egg white	21	2 c c 25% egg white	Exitus, typical shock	
30. 2 c c 5% egg white	25	2 c c 25% egg white	Exitus, typical shock	

* The sensitizing injections were given intraperitoneally, the intoxicating doses, intracardially. The various substances tested proved non-toxic for normal animals in the doses employed.

Anaphylaxis experiments with guinea-pigs served to confirm the lack of any antigenic properties possessed by the ¼, ⅓, ½ and ⅔ ammonium sulphate saturated fractions, and showed that the ¾ and 4/4 saturated products possessed slight sensitizing and intoxicating properties, the latter being apparently the more active.

In all forms of antibody production the antigenic proteoses were not specific, in that egg white could be used as antigen in place of either fraction when carrying out the tests. The reverse was not so marked.

(*c*) *Toxin-Antitoxin Reaction.*—According to Mitchell and Reichert[74] (1883) cobra venom contains 2% globulin which is the hemolysin and 98% of substances resembling "peptone," by which they probably meant substances now classed as proteoses. Antitoxin against cobra venom is quite efficient, and the possibility remains that we are dealing here with an anti-albumose. A further possibility is suggested, namely, are whole proteins necessary for anaphylaxis, precipitins and complement binding antibodies, while antitoxins are the result

[74] Cited by Wells, Chemical Pathology, 1918, p. 148.

of antigens consisting of derived proteins-albumoses? Weichardt[75] in connection with his studies of fatigue toxins (kenotoxins) obtained considerable evidence that the substances against which he was able to produce antitoxins represented the first stages in the disintegration of the protein molecule, and therefore closely related to proteoses.

One form of the epiphenin reaction reported by Weichardt[76] seemed to furnish a method of demonstrating the toxin-antitoxin reaction in vitro. He found that catalyzers (hemoglobin, platinum black) are markedly affected by bacterial toxins. If a water soluble toxin such as tetanus toxin be added to a much diluted blood, allowed to stand at 37 C. for 30 minutes, and then tincture of guaiac and hydrogen perox'd be added, the well known guaiac reaction does not occur. If the toxin is previously treated with its specific antitoxin there is no interference with the guaiac reaction and the solution turns blue. Nonspecific reactions may be avoided by quantitative means (dilution of antigen and serum). In addition to toxins, protein derived products including kenotoxin also inhibited the guaiac reaction.

I have test Weichardt's reaction, using the detailed protocol contained in his article. Diphtheria toxin and antitoxin, tetanus toxin and antitoxin, and all our proteose fractions and their corresponding serums were used. In the case of the bacterial toxins relatively large amounts of toxin were required for inhibition (500 units of diphtheria toxin and 1,000 of tetanus toxin). Much larger quantities of antitoxin were required to neutralize their action. The proteose preparations even as much as 0.1 gm., did not give the characteristic reaction. In my experience, the reaction was not of sufficient delicacy to be used in any sense as a quantitative test for the determination of toxin-antitoxin reactions.

SUMMARY

The results of these experiments indicate that Gibson's alcohol-soluble proteose obtained from Witte "peptone" is unable to stimulate the production of precipitins or complement binding antibodies when injected into rabbits. Two other fractions obtained from Witte "peptone" yielded similar results.

Anaphylaxis experiments with guinea-pigs showed that the Witte "peptone" preparations possessed only very slight power of sensitizing to themselves. They are not able to sensitize to beef serum, nor do they produce symptoms of anaphylactic intoxication in animals sensitized to beef serum. Beef serum did not sensitize to products of Witte "peptone," nor did it produce "shock" in animals sensitized to such products.

75. Ueber Ermüdungsstoffe, 1910.
76. Münch. med. Wchnschr., 1911, 58, p. 1667.

Proteose preparations were obtained by hydrolysis of coagulated egg white and fractional precipitation with ammonium sulphate in the manner described. There seems to be ground for disagreement with Haslam when he claims that constant nitrogen values as shown by Kjeldahl determinations are an index of purity of proteose preparations. A mixture of proteoses which is constant in its proportions will show constant quantities of nitrogen provided the proportions in the mixture remain the same. Kjeldahl nitrogen determinations of my preparations showed a maximum difference of less than 2% between all of them. When injected into dogs they showed the characteristic physiologic action of proteoses.

Experiments with rabbits indicated that the 1/4, 1/3, 1/2 and 2/3 saturation products possessed no power of stimulating the production of precipitins or complement binding antibodies. They also were unable to sensitize or intoxicate guinea-pigs either to themselves or to egg white, the mother protein.

The 3/4 and 4/4 saturation products were not as efficient antibody producers as egg white, but showed definite antigenic power for both precipitins and complement binding substances. In guinea-pigs the 3/4 and 4/4 saturated products possessed slight sensitizing and intoxicating properties, the latter being apparently the more active.

In all forms of antibody reaction the antigenic proteoses were not specific, in that egg white could be used as antigen in place of either fraction. The converse was not so marked.

Experiments with Weichardt's epiphanin reaction (interference with the guaiac blue reaction of blood) indicated that it is not sufficiently delicate to be used as a quantitative determination of the toxin-antitoxin reaction in vitro.

If the immune reactions are reliable indicators of chemical relationships, these results would seem to indicate that the 3/4 and 4/4 saturated fractions are chemically but little different from the mother substance, or at least contain similar antigenic groups.

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STUDIES ON PROTEINOGENOUS AMINES.

II. A MICROCHEMICAL COLORIMETRIC METHOD FOR ESTIMATING IMIDAZOLE DERIVATIVES.*

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INTRODUCTION.

The method described below is based upon the interaction between the imidazole ring and *p*-phenyldiazonium sulfonate (*p*-diazobenzene sulfonate).[1,2] Although this reaction has been used for a long time as a qualitative test for imidazoles there has been, as far as known to the authors, but one attempt made to apply it quantitatively. Weisz and Ssobolew[3] claim that a maximum color is obtained with 1 mg. of histidine dichloride under the conditions specified by them. Quantities of histidine either larger or smaller than 1 mg. give less intense colors even when the amount of *p*-phenyldiazonium sulfonate is increased. No attempt was made by the above authors to apply their method to other imidazoles. The method which we shall describe has, we believe, the following advantages over that of the above authors.

(*a*) It can be applied directly to practically any imidazole derivative.

(*b*) It gives equally good results on mixtures or pure solutions of imidazoles.

(*c*) As little as 0.00001 gm. of any of the imidazoles can be estimated with a fair degree of accuracy.

* The first article of this series was published in *J. Am. Chem. Soc.*, 1918, xl, 1716.

[1] Ehrlich, P., *Z. klin. Med.*, 1882, v, 285; *Char. Ann.*, 1883, viii, 140; *Deutsch. med. Woch.*, 1883, ix, 549; 1884, x, 419.

[2] Pauly, H., *Z. physiol. Chem.*, 1904, xlii, 508; 1905, xliv, 159.

[3] Weisz, M., and Ssobolew, N., *Biochem. Z.*, 1914, lviii, 119.

(*d*) A laborious series of dilutions is unnecessary. The first determination is usually a reliable index of the quantity of imidazole present.

In the ordinary qualitative procedure suggested by Ehrlich[1] and by Pauly[2] the imidazole solution is mixed first with the acid *p*-phenyldiazonium sulfonate reagent and this solution is then treated with an excess of a base, either sodium carbonate or ammonium hydroxide. We have found it necessary to mix the *p*-phenyldiazonium sulfonate solution first with the alkali sodium carbonate and then to add the imidazole-containing solution to this alkaline liquid to obtain strictly quantitative color productions. The necessity for such a change in procedure is readily seen when the chemical reactions involved are considered.

It is generally conceded that the first reaction proceeds according to the following equation.

$$(1)\quad HO-SO_2-C_6H_4-\underset{\displaystyle Cl}{\underset{|}{NH_3}} + HNO_2 \rightarrow HO-SO_2-C_6H_4-\underset{\displaystyle Cl}{\underset{|}{N}}\equiv N + 2H_2O$$

By allowing a mixture containing an excess of hydrochloric and nitrous acids to react for 5 minutes at 0°, one is assured of a complete conversion of the sulfanilic acid into the diazonium salt.

After the above reaction has occurred, an excess of sodium nitrite is added to the liquid which brings about an immediate decrease in the concentration of the hydrogen ions with the formation of sodium chloride and nitrous acid.

The work of Hantzsch,[4] Goldschmidt and his coworkers,[5] von Pechmann,[6] and many others has shown that:

1. Diazonium salts are similar to ammonium salts; hence the

[4]A summary of the elaborate researches on the subject of diazonium salts may be found in Hantzsch, A., Die Diazoverbindungen, Stuttgart, 1902.

[5] Goldschmidt, H., and Merz, A., *Ber. chem. Ges.*, 1897, xxx, 670. Goldschmidt, H., and Buss, F., *ibid.*, 1897, xxx, 2075. Goldschmidt, H., and Bürkle, E., *ibid.*, 1899, xxxii, 355. Goldschmidt, H., and Keppeler, G., *ibid.*, 1900, xxxiii, 893. Goldschmidt, H., and Keller, H., *ibid.*, 1902, xxxv, 3534.

[6] von Pechmann, H., *Ber. chem. Ges.*, 1892, xxv, 3505. von Pechmann, H., and Frobenius, L., *ibid.*, 1894, xxvii, 651.

salt of a diazonium base with a weak acid, like nitrous acid, will be hydrolyzed by water to a marked extent.

2. Diazonium hydroxides pass into diazohydrates at a rapid but measurable rate of speed.

3. The speed of coupling is directly proportional to the concentration of the diazohydrate (or its nitrosamine isomer). Thus Goldschmidt and his coworkers found that the rate of coupling of diazonium salts depended upon the degree of hydrolysis (not ionization) of the salt and not upon the concentration of the diazonium salt present. Similarly the rate of coupling of the alkali diazotates was found to be proportional to their degree of hydrolysis. Goldschmidt's work does not show, however, whether it is the diazohydrate or the nitrosamine derivative that is responsible for the coupling.

4. Compounds of unquestionable nitrosamine structure such as acetylphenylnitrosamine (nitrosoacetanilide) $C_6H_5\text{-}N(NO)\text{-}CO.CH_3$ couple readily with aromatic amines to give diazoamino compounds and with phenols to give oxyazo compounds, acetic acid being eliminated.[6] The compounds so obtained are identical with those formed with the corresponding diazonium salts.

$$\begin{array}{l} C_6H_5-\underset{\displaystyle NO}{\underset{|}{N}}-CO-CH_3 \end{array} + H_2N-C_6H_4-CH_3 \rightarrow C_6H_5-N:N-\underset{\displaystyle H}{N}-C_6H_4-CH_3 + CH_3-COOH$$

$$C_6H_5-\underset{\displaystyle N}{\underset{|||}{N}}-Cl + H_2N-C_6H_4-CH_3 \rightarrow C_6H_5-N:N-\underset{\displaystyle H}{N}-C_6H_4-CH_3 + HCl$$

This fact has lead us to believe that it is the nitrosamine that is the active coupling agent and not the diazohydrate.

The series of changes that occur when an excess of sodium nitrite is added to a solution containing the diazonium salt of sulfanilic acid can be formulated as follows.

$$(2)\quad HO-SO_2-C_6H_4-N_2Cl+2NaNO_2 \underset{(\alpha)}{\rightleftarrows} NaO-SO_2-C_6H_4-N_2-O-N:O+NaCl+HNO_2$$

$$(3)\quad NaO-SO_2-C_6H_4-\underset{\displaystyle N}{\underset{|||}{N}}-O-N:O + H_2O \rightleftarrows NaO-SO_2-C_6H_4-\underset{\displaystyle N}{\underset{|||}{N}}-OH + HNO_2$$

$$\downarrow\uparrow \quad (\beta)$$

$$(B)\quad NaO-SO_2-C_6H_4-\underset{\displaystyle N=O}{\underset{|}{N}}-H \rightleftarrows NaO-SO_2-C_6H_4-\underset{\displaystyle HO-N}{\underset{\|}{N}} \quad (A)$$

The finished solution, after equilibrium has been established, should contain considerable of the compounds (A) and (B) either or both of which are the active coupling agents (see formulas). We have found it necessary to allow the finished diazo reagent to stand in an ice bath for at least 15 minutes before using to get uniform results.

The above solution contains variable amounts of nitrous acid, variable because some of it escapes in the form of anhydride whenever the containing flask is opened. If a substance containing an aliphatic amino group, such as histidine, is now added to the above solution, which is the qualitative procedure usually followed, variable amounts of this amino group are replaced by the hydroxyl radical. The colors produced by the hydroxy compounds are, however, not identical with those obtained from the amino compounds and, therefore, a given amount of histidine will not always give exactly the same color under the conditions prescribed for the qualitative test, since the per cent of deamination is different in each case.

If the diazo reagent were *first* mixed with an *alkali* before adding the imidazole solution, two reactions would occur: (1) the free nitrous acid would be entirely neutralized, so there could be no disruption of the aliphatic amino groups; and (2) the equilibrium represented in Equation 3 would be displaced to the right which would lead to an increase in the concentration of the active coupling compounds (A) and (B) or to the formation of the disodium salt of (A).

$$\begin{array}{r} Na-O-SO_2-C_6H_4-N \\ \| \\ Na-O-N \end{array}$$

The disodium salt represented above would be rapidly hydrolyzed by water;[9] so we would not expect it to be formed in quantity unless an excess of strong alkali, such as sodium hydroxide, were added to the solution. With a properly chosen alkali, the completed solution should contain a uniform number of active coupling molecules which should then give identical colors with the same quantity of imidazole. By a strict adherence to the directions given, which were based upon the above theoretical deductions, we have found it possible to estimate imidazole derivatives with an accuracy equivalent to that of any colorimetric method.

Sodium carbonate has been found to be the ideal alkali and it was used in preference to any other because of the following facts: (*a*) with sodium acetate no color is obtained; (*b*) sodium bicarbonate gives a color only when the bicarbonate is present in large excess (even then the color appears very slowly and is lacking in intensity); (*c*) ammonium hydroxide cannot be used because, as will be shown later, it gives a green color with diazotized sulfanilic acid; (*d*) in the presence of sodium hydroxide, weak yellow to pale orange, very labile colors are produced.

In searching for a color standard against which to match the colors produced by the reaction, it was of course natural to seek first for stable, chemically allied substances. Both methyl orange and Congo red are chemically related to the colored substances produced with the imidazoles, and we have found that Congo red alone, or mixtures of it with methyl orange do indeed give the desired colors. Combinations of these indicators ought to prove useful as color standards in other cases where the colors produced are yellow to red.

EXPERIMENTAL.

Reagents Employed.

Stock Sulfanilic Acid.—Sulfanilic acid (4.5 gm.) is dissolved in 45 cc. of 37 per cent hydrochloric acid (sp. gr. 1.19) in a 500 cc. volumetric flask and water added to the mark.

Stock Sodium Nitrite.—25 gm. of 90 per cent sodium nitrite are dissolved in water and diluted to 500 cc. in a volumetric flask.

Sodium Carbonate.—Merck's or J. T. Baker's anhydrous sodium carbonate (5.50 gm.) is dissolved in water and diluted to exactly 500 cc. We specify the above two grades of carbonate because they were found to give uniform results. Two other brands tried by us were found to give yellow colors that were quite different in shade from those produced with the specified salts. The finished carbonate solution must be preserved in a glass vessel that has little tendency to dissolve in alkali. Pyrex glass vessels have proved to be entirely satisfactory.

Stock Methyl Orange.—Vacuum-dried Grübler's methyl orange (0.5000 gm.) is dissolved in water and diluted to exactly 500 cc. This solution keeps indefinitely.

Stock Congo Red.—Vacuum-dried Grübler's Congo red (2.5000 gm.) is mixed with 50 cc. of absolute alcohol in a 500 cc. volumetric flask. Water is then added to the mark. This solution keeps indefinitely.

Standard Indicator Solutions.—For the estimation of *imidazole acetic, imidazole propionic acid*, and *methyl imidazole*, a solution containing 1 cc. of the stock Congo red solution in a total aqueous volume of 500 cc. is employed. When this standard indicator solution has been used for comparisons the symbol (CR) is suffixed to the reading obtained.

For the estimation of *histidine* or *histamine*, 1 cc. of stock Congo red and 1.1 cc. of stock methyl orange are allowed to flow into 250 cc. of water contained in a 500 cc. volumetric flask. Water is then added to the mark. The stock indicator solutions must not be mixed in concentrated form. When this is done a rapid interaction seems to occur with destruction of color.

When this standard indicator solution has been used for comparisons the symbol (CR-MO) is suffixed to the reading obtained. When this solution is properly prepared and preserved in a tightly stoppered flask, it can be kept with certainty for 2 weeks. It is best to keep this solution in a hard glass vessel, such as Pyrex, because the alkali introduced by the solution of a soft glass changes the color of the indicators enough to give untrustworthy results.

Preparation of p-Diazobenzene Sulfonic Acid Solution. (*The Reagent*).—1.5 cc. each of the stock sulfanilic acid and sodium nitrite solutions are measured into a 50 cc. volumetric flask. The flask is then immersed in an ice bath for 5 minutes. Then 6 cc. more of the stock sodium nitrite solution are added and the well-mixed solution again allowed to lie in the ice bath for 5 minutes. Distilled water is then added to the mark and the flask returned to the ice bath where it is kept. This reagent must not be used for at least 15 minutes after diluting with water. We have found it to give perfect results after 24 hours. It is best, however, to prepare a fresh reagent every day.

Preparation of the Imidazoles.

Histidine Dichloride.—This was prepared according to a slight modification of the method of Fränkel.[7] Its purity was determined by analysis after it had been dried for 48 hours in vacuum.

Ammonia was absent.

It melted with gaseous decomposition at 248°.

0.06012 gm. gave 6.52 and 6.62 cc. of nitrogen gas at 20° and 750 mm., by the Van Slyke method.

	Calculated.	Found.	
	per cent	*per cent*	*per cent*
Amino nitrogen................	6.14	6.09	6.18

The chloride ion from 0.3009 gm. of substance was completely precipitated by 26.16 cc. of 0.1 N $AgNO_3$. This quantity of 100 per cent histidine dichloride should have used 26.40 cc.

The above two analyses prove that the histidine dichloride used was 100 per cent pure.

[7] Fränkel, S., *Monatsh. Chem.*, 1903, xxiv, 230.

Histamine Dichloride.—The method of preparation and analysis of this compound have been previously described by us.[8]

Imidazole Propionic Acid.—Glyoxal propionic acid was prepared according to Wolff.[9] From it the imidazole propionic acid was then prepared according to Knoop and Windaus.[10] After recrystallizing the final product three times from 75 per cent acetone, too little was left to make a very exhaustive analysis. After drying in vacuum over sulfuric acid for 48 hours the pure white crystalline solid had the following properties.

It melted at 207–209°.

Ammonia was absent.

No residue on ignition.

The substance was assumed to be 100 per cent pure imidazole propionic acid.

Imidazole Acetic Acid Hydrochloride.—The substance used was obtained as a by-product in the preparation of histamine dichloride and its mode of isolation has been previously described.[11] After drying in vacuum over sulfuric acid for 48 hours the pure white, glistening solid had the following properties.

It melted at 226–228° (corrected).

Ammonia was absent.

No residue on ignition.

The chloride ion was completely precipitated from 0.1625 gm. of substance—0.10 mol—by 10.05 cc. of 0.1 N $AgNO_3$. This quantity of 100 per cent hydrochloride should have used 10.00 cc. The above properties prove that the substance used was 100 per cent pure.

4-Methyl Imidazole.—This substance was prepared from commercial glucose, ammonia, and zinc hydroxide. The directions given by Windaus and Knoop[12] were followed with the following modifications.

The solid zinc methyl imidazole hydroxide was dissolved in ten times its weight of water to which an excess of acetic acid had been added. The mixture was saturated with hydrogen sulfide under pressure and filtered from precipitated zinc sulfide. The resulting brown solution was distilled in vacuum at 50° to remove the water, hydrogen sulfide, and acetic acid. The residual dark brown gum was then extracted with ten times its weight of cold dry acetone which dissolved the methyl imidazole completely but left practically all the coloring matter behind. The oxalate was then precipitated by adding a solution of oxalic acid in acetone to the above acetone solution of methyl imidazole. The oxalate is soluble in dry acetone only in traces. The nearly white solid was then recrystallized from 75 per cent acetone until it melted at 206°. The free base was prepared

[8] Koessler, K. K., and Hanke, M. T., *J. Am. Chem. Soc.*, 1918, xl, 1716.

[9] Wolff, L., *Ann. Chem.*, 1885, ccxxix, 266.

[10] Knoop, F., and Windaus, A., *Beitr. chem. Physiol. u. Path.*, 1906, vii, 144.

[11] Hanke, M. T., and Koessler, K. K., *J. Am. Chem. Soc.*, 1918, xl, 1726.

[12] Windaus, A., and Knoop, F., *Ber. chem. Ges.*, 1905, xxxviii, 1166.

from the oxalate by the method of Windaus and Knoop, and was obtained first as a colorless mobile oil, and then as a colorless solid which had the following properties.

It melted at 56.5°.

Ammonia was absent.

No residue on ignition.

0.2000 gm. was dissolved in 5 cc. of water and titrated with 0.1 N hydrochloric acid using dimethylaminoazobenzene as indicator. Exactly 24.40 cc. of acid were required to produce the first change in the indicator which is exactly the amount demanded for this quantity of 100 per cent methyl imidazole. The substance was, therefore, 100 per cent pure.

Procedure for Estimating Imidazoles.

The method used in developing the tables as well as the general procedure for estimating imidazoles is illustrated by the following example.

$(1-X)$ cc. of water and 5 cc. of the 1.1 per cent sodium carbonate solution are accurately measured into the right hand cylinder of a Duboscq colorimeter. 2 cc. of reagent are measured into a 5 seconds delivery 2 cc. pipette, the time noted to the second, and the reagent allowed to flow into the alkali. The contents of the cylinder are then thoroughly mixed by allowing the liquid to flow repeatedly up the inclined tube as far as safety from loss will permit. The mixing should not take over 30 seconds. X cc. of the imidazole solution are allowed to flow into the cylinder exactly 1 minute after the reagent began to mix with the alkali. The contents of the cylinder are mixed *thoroughly* as above. The test cylinder is then transferred to the colorimeter and set at 20 mm. The right hand cylinder which should contain the appropriate standard indicator solution is then adjusted constantly until a maximum reading has been obtained.[13]

The speed of color formation varies with the different imidazoles as will be made clear when the individual tables are discussed. In every case, however, a maximum color intensity is reached in the course of 5 to 10 minutes and at this point the color remains stationary for a period of from 1 to 10 minutes depending upon the imidazole used. Histamine gives a promptly appearing

[13] This is the inverse of the process as it is ordinarily carried out where the standard solution is set at some definite value and the test cylinder moved until a match is obtained.

labile color whose duration of maximum intensity is about 1 minute. With histidine the color development is gradual, takes about 6 minutes to reach its maximum intensity, and lasts from 2 to 3 minutes. In every case the weaker colors are the more stable. The most accurate determinations can be obtained by choosing such an amount of imidazole solution that the standard indicator cylinder has to be set at from 5 to 20 mm.

The method described here can be used on quantities of the imidazole-containing solution varying from 0.01 to 1.0 cc. The combined volume of water and imidazole solution used should always be 1 cc. Thus if 0.10 cc. of the imidazole solution is to be used, 0.90 cc. of water is added to the test cylinder. Then X equals 0.10 cc. and $1-X$ equals 0.90 cc.

Estimation of Small Amounts of Histidine.

A stock histidine solution was prepared by dissolving 2.000 gm. of 100 per cent histidine dichloride in water and diluting to exactly 200 cc. A layer of toluene was added as a preservative. From this the standard solution was prepared by diluting 1 cc. of stock solution to 100 cc. Each cc. of the standard solution contained 0.0001 gm. of histidine dichloride.

The color develops slowly in this case. At first a yellow color is produced which changes rapidly to orange and then more slowly to pink. A maximum color value is obtained after 5 to 6 minutes, and is maintained in the weaker solutions for about 3 minutes. Then the color changes slowly through orange to yellow.

The previously described (CR-MO) indicator was used for comparisons. The color generated by histidine contains slightly more yellow than the standard indicator solution but the difference in shade is so slight, that with practice, an intensity comparison can be made with an accuracy of from 0.5 to 3 per cent, as has been demonstrated by the determination of unknowns. Where histidine is the only substance to be determined a more perfect color match can be obtained by using more of the stock methyl orange solution in compounding the standard indicator solution. If this is done, however, Table I cannot be used. We have preferred to use this standard indicator solution because, as will be shown in the following two articles, it is more universally applicable to mixtures of imidazoles than any other.

TABLE I.

Estimation of Small Amounts of Histidine.

Depth of indicator solution (CR-MO) required to match the color in the test cylinder.	Histidine dichloride in the test cylinder (total volume 8 cc.). Test cylinder set at 20 mm.
mm.	*gm.*
0.5	0.000001
1.0	0.000002
1.5	0.000003
2.0	0.000004
2.5	0.000005
3.0	0.000006
3.5	0.000007
4.0	0.000008
4.5	0.000009
5.0	0.000010
5.5	0.000011
6.0	0.000012
6.5	0.000013
7.0	0.000014
7.5	0.000015
8.0	0.000016
8.5	0.000017
9.0	0.000018
9.5	0.000019
10.0	0.000020
10.5	0.000021
11.0	0.000022
11.5	0.000023
12.0	0.000024
12.5	0.000025
13.0	0.000026
13.5	0.000027
14.0	0.000028
14.5	0.000029
15.0	0.000030
15.5	0.000031
16.0	0.000032
16.5	0.000033
17.0	0.000034
17.5	0.000035
18.0	0.000036
18.5	0.000037
19.0	0.000038
19.5	0.000039
20.0	0.000040

TABLE I (Continued).

Depth of indicator solution (CR-MO) required to match the color in the test cylinder.	Histidine dichloride in the test cylinder (total volume 8 cc.). Test cylinder set at 20 mm.
mm.	*gm.*
20.5	0.000041
21.0	0.000042
21.5	0.000043
22.0	0.000044
22.5	0.000045
23.0	0.000046
23.5	0.000047
24.0	0.000048
24.5	0.000049
25.0	0.000050

Table I shows clearly that the color production is directly proportional to the quantity of histidine dichloride used. This fact can be conveniently represented by the formula $I \times 0.000002 = H$, where I is the depth of the indicator solution in mm. required to match the color in the test cylinder, and H is the number of gm. of histidine dichloride in the test cylinder. Thus if the indicator solution had to be set at 18.5 mm. to match the color in the test cylinder, I would equal 18.5 and H would be equal to $18.5 \times 0.000002 = 0.000037$ gm. of histidine dichloride in the test cylinder.

Estimation of Small Amounts of Histamine (β-Imidazolylethylamine).

A stock histamine solution was prepared by dissolving 2.1097 gm. of 94.8 per cent histamine dichloride in water and diluting to exactly 200 cc. A layer of toluene was added as a preservative. From this the standard solution was prepared by diluting 1 cc. of the stock solution to 100 cc. Each cc. of the standard solution contained 0.0001 gm. of histamine dichloride.

The color develops quite rapidly in this case and becomes pink almost immediately. Although most of the color develops within 1 minute, a maximum is reached only after 4 to 5 minutes. With dilute solutions—0.00002 gm. or less—the color of maximum intensity persists for about 2 minutes. With more concentrated solutions, the period of maximum intensity may be as short as 30 seconds.

TABLE II.

Estimation of Small Amounts of Histamine.

Depth of indicator solution (CR-MO) required to match the color in the test cylinder.	Histamine dichloride in the test cylinder (total volume 8 cc.). Test cylinder set at 20 mm
mm.	*gm.*
0.8	0.000001
1.5	0.000002
2.2	0.000003
3.0	0.000004
3.7	0.000005
4.5	0.000006
5.2	0.000007
6.0	0.000008
6.7	0.000009
7.5	0.000010
8.2	0.000011
9.0	0.000012
9.7	0.000013
10.5	0.000014
11.2	0.000015
12.0	0.000016
12.7	0.000017
13.5	0.000018
14.2	0.000019
15.0	0.000020
15.7	0.000021
16.5	0.000022
17.2	0.000023
18.0	0.000024
18.7	0.000025
19.5	0.000026
20.2	0.000027
21.0	0.000028
21.7	0.000029
22.5	0.000030
23.2	0.000031
24.0	0.000032
24.7	0.000033
25.5	0.000034
26.2	0.000035
27.0	0.000036
27.7	0.000037
28.5	0.000038
29.2	0.000039
30.0	0.000040

TABLE II—*Concluded.*

Depth of indicator solution (CR-MO) required to match the color in the test cylinder.	Histamine dichloride in the test cylinder (total volume 8 cc.). Test cylinder set at 20 mm.
mm.	*gm.*
30.7	0.000041
31.5	0.000042
32.2	0.000043
33.0	0.000044
33.7	0.000045
34.5	0.000046
35.2	0.000047
36.0	0.000048
36.7	0.000049
37.5	0.000050

The previously described (CR-MO) indicator matches the color produced by histamine perfectly.

From Table II the facts can be represented by a formula, namely,

$$I \times 0.00001333 \ldots = H_s$$

where H_s is the number of gm. of histamine in the test cylinder and I retains the meaning assigned to it under Table I.

Estimation of Small Amounts of Imidazole Propionic Acid.

A stock solution was prepared by dissolving 2.0000 gm. of 100 per cent imidazole propionic acid in water, adding enough of a normal solution of hydrochloric acid to convert it into the hydrochloride, and finally diluting with water to 200 cc. A few drops of chloroform were added as a preservative. From this the standard solution was prepared by diluting 1 cc. of the stock solution to 100 cc. Each cc. of the standard solution contained 0.0001 gm. of imidazole propionic acid.

A large per cent of the color develops immediately and it is red from the beginning. An accurate comparison with the indicator can be made at the end of 1 minute. Thus it was found that for 0.00001 gm. of substance a color value of 13.0 mm. (CR) was obtained at the end of 1 minute and 15.3 mm. at the end of 10 minutes which is the time required to give a maximum color at 20°. The rate of development depends to a large extent upon the temperature. Thus at 18°, 13 minutes were required to give

TABLE III.

Estimation of Small Amounts of Imidazole Propionic Acid.

Depth of indicator solution (CR) required to match the color in the test cylinder.	Imidazole propionic acid in the test cylinder (total volume 8 cc.). Test cylinder set at 20 mm.
mm.	*gm.*
1.5	0.000001
3.0	0.000002
4.5	0.000003
6.0	0.000004
7.5	0.000005
9.0	0.000006
10.5	0.000007
12.0	0.000008
13.5	0.000009
15.0	0.000010
16.5	0.000011
18.0	0.000012
19.5	0.000013
21.0	0.000014
22.5	0.000015
24.0	0.000016
25.5	0.000017
27.0	0.000018
28.5	0.000019
30.0	0.000020
31.5	0.000021
33.0	0.000022
34.5	0.000023
36.0	0.000024
37.5	0.000025
39.0	0.000026
40.5	0.000027
42.0	0.000028
43.5	0.000029
45.0	0.000030

a maximum of color while at 22° only 8 minutes were required. The *amount* of color finally produced is always the same regardless of the temperature. The color is unusually stable. After reaching its maximum there is no apparent change for 5 minutes.

The previously described (CR) indicator matches the color produced by imidazole propionic acid perfectly.

Table III was not extended because the colors were too intense to make an accurate comparison possible. The values higher than 25 mm. of indicator solution were obtained by setting the test cylinder at 10 instead of 20 mm. and multiplying the reading obtained by 2. The facts can in this case be represented by the formula $I \times 0.0000006666 \ldots = P$ where P is the number of gm. of imidazole propionic acid in the test cylinder.

Estimation of Small Amounts of Imidazole Acetic Acid.

A stock solution was prepared by dissolving 2.0000 gm. of 100 per cent imidazole acetic acid hydrochloride in water and diluting to exactly 200 cc. A few drops of chloroform were added as a preservative. From this the standard solution was prepared by diluting 1 cc. of the stock solution to 100 cc. Each cc. of the standard solution contained 0.0001 gm. of the hydrochloride of imidazole acetic acid.

The (CR) indicator matches the color produced by imidazole acetic acid perfectly. Table IV shows the results of the test.

The statements given above for imidazole propionic acid apply equally well in this case. The facts can be represented by the formula $I \times 0.00000081308 = A$, where A is the number of gm. of imidazole acetic acid in the test cylinder.

Estimation of Small Amounts of Methyl Imidazole.

A stock solution was prepared by dissolving 2.0000 gm. of the base in 24.4 cc. of N HCl and diluting with water to exactly 200 cc. A few drops of chloroform were added as a preservative. From this the standard solution was prepared by diluting 1 cc. of the stock solution to 100 cc. Each cc. of the standard solution contained 0.0001 gm. of methyl imidazole.

A large per cent of the color develops immediately which is red and comparable with the indicator from the start. The color reaches its maximum intensity in 7 minutes at 20°, and undergoes no apparent change for 15 minutes. The (CR) indicator was used for comparisons. The color match is not quite perfect in this case, as the color produced by the methyl imidazole is slightly too red for the (CR) indicator. The discrepancy in shade is so slight, however, that an accurate comparison can easily be made.

TABLE IV.

Estimation of Small Amounts of Imidazole Acetic Acid.

Depth of indicator solution (CR) required to match the color in the test cylinder.	Imidazole acetic acid hydrochloride in the test cylinder (total volume 8 cc.). Test cylinder set at 20 mm.
mm.	*gm.*
1.2	0.000001
2.5	0.000002
3.7	0.000003
4.9	0.000004
6.1	0.000005
7.4	0.000006
8.6	0.000007
9.8	0.000008
11.1	0.000009
12.3	0.000010
13.5	0.000011
14.7	0.000012
16.0	0.000013
17.2	0.000014
18.5	0.000015
19.7	0.000016
20.9	0.000017
22.1	0.000018
23.4	0.000019
24.6	0.000020
25.8	0.000021
27.0	0.000022
28.3	0.000023
29.5	0.000024
30.7	0.000025
32.0	0.000026
33.2	0.000027
34.4	0.000028
35.7	0.000029
36.9	0.000030
38.1	0.000031
39.4	0.000032
40.6	0.000033
41.9	0.000034
43.0	0.000035
44.3	0.000036
45.5	0.000037
46.7	0.000038
47.9	0.000039
49.2	0.000040

Table V was not extended beyond 0.00002 gm. of methyl imidazole because the colors were too intense to make an accurate comparison possible. The values higher than 25 mm. of indicator solution were obtained by setting the test cylinder at 10 instead of 20 mm. and multiplying the reading obtained by 2. The facts can be represented by the formula $I \times 0.00000037037 \ldots = M$ where M is the number of gm. of methyl imidazole in the test cylinder.

TABLE V.

Estimation of Small Amounts of Methyl Imidazole.

Depth of indicator solution (CR) required to match the color in the test cylinder.	Methyl imidazole in the test cylinder (total volume 8 cc.). Test cylinder set at 20 mm.
mm.	*gm.*
2.7	0.000001
5.4	0.000002
8.1	0.000003
10.8	0.000004
13.5	0.000005
16.2	0.000006
18.9	0.000007
21.6	0.000008
24.3	0.000009
27.0	0.000010
29.7	0.000011
32.4	0.000012
35.1	0.000013
37.8	0.000014
40.5	0.000015
43.2	0.000016
45.9	0.000017
48.6	0.000018
51.3	0.000019
54.0	0.000020

Relative Value of the Standard Indicator Solutions.

The relative intensity of the two standard indicator solutions can be compared by means of the Duboscq colorimeter even though the tint is not identical. This enables one to compare readings obtained with the (CR) indicator with those obtained with the (CR-MO) indicator. The readings obtained, representing mm. on the Duboscq scale, are as follows.

(CR) standard indicator.	(CR-MO) standard indicator.
11	10
22	20
33	30

The following example will illustrate the use of the table. 0.00001 gm. of the hydrochloride of glyoxaline acetic acid has a color value on the (CR) scale of 12.3 mm. by actual determination. A calculation from the table shows that the color value on the (CR-MO) scale should be $\frac{10}{11} \times 12.3 = 11.2$ mm. The actual determination using the (CR-MO) scale, which was made fully 2 months before this comparison table was compiled, actually gave 11.2 mm. It is now possible to compare the molecular color values of the various imidazoles.

Molecular Color Value of the Five Imidazoles.

The following figures were obtained by calculating the hypothetical color value on the (CR-MO) scale that would be obtained if one molecular weight of the imidazole had been present in the test cylinder. The calculations are based on the values recorded in the tables for 0.00001 gm. of the imidazole derivatives.

	Weight. *mol*	Molecular color value.
Histidine dichloride	228.0	114,000,000
Histamine "	184.0	138,000,000
Imidazole propionic acid	140.0	191,000,000
" acetic acid as HCl	162.5	182,000,000
Methyl Imidazole	82.0	201,000,000

From this tabulation the following conclusions can be drawn.

1. The color produced by these five imidazoles depends not only upon the imidazole ring but is strongly influenced by the groups in the side chain also.

2. The color is nearly a pure red and of maximum intensity when the side chain is paraffin in character (methyl imidazole).

3. The carboxyl group makes the color more yellow and reduces its intensity. This effect is most marked when the carboxyl group is near the imidazole ring. Thus imidazole acetic acid has a molecular color value that is about 90 per cent as great as that of methyl imidazole, whereas imidazole propionic acid has a molecular color value that is 95 per cent as great as that of methyl imidazole.

4. The introduction of an amino group greatly reduces the intensity of the color and makes it decidedly yellow. When both the amino and the carboxyl group are present in the same molecule, the effect on the color is additive. Thus histamine has a molecular color value that is about 69 per cent as great as that of the methyl imidazole whereas histidine has a molecular color value that is only 57 per cent as great as that of methyl imidazole.

The Correction Blank.

When the reagent and alkali are mixed in the absence of an imidazole derivative, a very pale yellow color is produced in the course of 5 to 10 minutes that has an intensity value of 0.30 mm. (CR-MO). This same quantity of color is also produced in the presence of imidazole derivatives along with the color produced by the imidazole; so the readings obtained are too high by 0.30 mm. in every case. It is necessary, therefore, to subtract 0.30 mm. from the readings obtained before comparison with the tables. For example, 0.10 cc. of a histidine solution is found to have a color value equivalent to 10.3 mm. (CR-MO). The corrected reading is 10.0 mm. (CR-MO); so 0.10 cc. of this solution contains 0.00002 gm. of histidine dichloride (Table I). It is hardly necessary to say that the correction blank will be 0.30 mm. only when the test cylinder is set at 20 mm.

Substances That Do and Do Not Interfere with the Quantitative Determination of Histidine.

2 cc. of stock histidine solution were mixed in a 100 cc. precision cylinder with quantities of the other substances as stated in Table VI. Water was then added to give a final volume of 100 cc. Then 0.10 cc. of the solution was taken for the colorimetric determination.

The most active interfering substances are apparently ketones, alcohols, and ammonium salts. The volatile ketones and alcohols can always be removed by evaporation; so they offer no permanent difficulties. Amyl alcohol can be completely removed from an aqueous solution by extraction with toluene or chloroform. The resulting aqueous solution gives the correct color value. The

dissolved chloroform or toluene does not interfere with the production of color. Glycerol or glucose is seldom present, in concentrations as high as those given here, in the usual biological liquids.

TABLE VI.

Substance.	Concentration	Color.	Interference.
	per cent		
Urea.	5	Perfect.	None.
NaCl KCl Na_2SO_4 KH_2PO_4	5	"	"
Sodium acetate. Sodium citrate.	5	"	"
Glycerol.	3	"	2 per cent low in color value.
Glucose.	5	Slightly too yellow.	8 " " " " " "
Ethyl alcohol.	5 10	Perfect.	5 " " " " " " 10 " " " " " "
Methyl alcohol.	10	Slightly too yellow.	20 " " " " " "
Amyl alcohol.	Saturated aqueous solution.	" "	10 " " " " " "
Acetone.	10	Greenish yellow.	Entirely unmatchable color of slight intensity. No determination possible.
$(NH_4)_2SO_4$ free from pyridine.	1 5	Slightly brown. Greenish "	2 per cent high in color value. Entirely unmatchable color of high intensity. No determination possible.

The following substances require individual consideration.

Ammonium salts.—The positive interference referred to above for ammonium sulfate led us to believe that ammonia itself gave

a color with the reagent. Experiment proved this to be the case. A 10 per cent aqueous solution of the highest purity of ammonium sulfate obtainable, which was free from pyridine, was tested for color value by introducing 0.10 cc. of it into the test cylinder in the usual manner. A fairly intense yellowish green color developed promptly. Exactly the same quantity and quality of color were obtained when 0.50 cc. of the ammonium sulfate solution was tested.

The color value of a solution containing more than the equivalent of 1 per cent of ammonium sulfate is untrustworthy.

Proteins.—The colorimetric method is worthless when applied to a solution containing soluble proteins for two reasons. (*a*) The proteins themselves give a color with the reagent which is not quantitatively produced. Thus 0.20 cc. of a protein solution gives less than twice the color produced by 0.10 cc. (*b*) The addition of a known amount of histidine to a solution containing protein of previously determined color value gives less than the theoretical increase in color value. The results with blood proteins run about 30 per cent low.

It might be possible to eliminate this protein interference by using a stronger reagent. This would make it necessary to develop a new set of tables. No experiments have been made up to the present time to verify the correctness of the above statement.

Animal Charcoal.—We have found that animal charcoal cannot be used to decolorize a solution in which the presence of small amounts of imidazoles is to be determined, because the charcoal adsorbs appreciable quantities of these substances. Experimental data are given in Table VII to support the above statement. Kahlbaum's highest grade of powdered bone charcoal was used in these experiments. A weighed quantity of the charcoal was agitated for 10 minutes with 100 cc. of the imidazole solution. The color value of the clear solution was then determined.

Table VII shows clearly that for preparative work, or even for quantitative work where the concentration of the imidazole is high, charcoal might be used with impunity because the maximum adsorption per gm. of charcoal seems to be about 1 mg. of imidazole. It is best, however, to avoid the use of charcoal when possible.

Barium sulfate precipitates do not adsorb imidazoles.

The general method outlined will undoubtedly be found to be equally applicable to the estimation of all imidazoles with the possible exception of cyanomethyl imidazole which gives a brown and not an orange or red color. It is also applicable to the estimation of other compounds that will couple with *p*-diazobenzene sulfonic acid such as phenols and it may prove useful for the estimation of aromatic amines. We hope to report the method for estimating phenols in the near future.

TABLE VII.

Charcoal added.	Amount and kind of imidazole used (total volume 100 cc.).	Color value of indicator solution before adding charcoal.	Color value of indicator solution after adding charcoal.	Imidazole adsorbed by the charcoal.	
gm.	*gm.*	*mm.*	*mm.*	*gm.*	*per cent*
1	0.01 methyl imidazole.	0.10 cc. equivalent to 27.3 mm. (CR).	0.10 cc. equivalent to 9.5 mm. (CR).	0.0066	66
2	0.01 methyl imidazole.	0.10 cc. equivalent to 27.3 mm. (CR).	0.10 cc. equivalent to 5.5 mm. (CR).	0.0080	80
4	0.01 methyl imidazole.	0.10 cc. equivalent to 27.3 mm. (CR).	0.10 cc. equivalent to 3.0 mm. (CR).	0.0090	90
1	0.02 methyl imidazole.	0.10 cc. equivalent to 54.3 mm. (CR).	0.10 cc. equivalent to 33.5 mm. (CR).	0.0075	37.5
1	0.01 histamine dichloride.	0.20 cc. equivalent to 15.3 mm. (CR-MO).	0.20 cc. equivalent to 2.0 mm. (CR-MO).	0.0090	90

Our interest in these particular imidazoles will be made clear in the succeeding two papers.

SUMMARY.

1. The well known interaction between the imidazole ring and *p*-phenyldiazonium sulfonate has been made the basis of a quantitative colorimetric method for estimating histidine, histamine,

imidazole propionic acid, imidazole acetic acid, and methyl imidazole.

2. Tables are given for the direct determination of quantities of these substances ranging from 0.000001 to 0.00005 gm. The amount of imidazole in any quantity of liquid can then be determined, by multiplication, with an accuracy of from 0.5 to 3 per cent.

3. A list of substances is given, some of which do and others of which do not interfere with the determination.

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STUDIES ON PROTEINOGENOUS AMINES.

III. A QUANTITATIVE METHOD FOR SEPARATING HISTAMINE FROM HISTIDINE.

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An exact study of the metabolism of histidine has been very difficult up to the present time because we do not have the proper quantitative method for estimating imidazole derivatives. It has always been necesssary, therefore, either to isolate the imidazole derivative or to determine its presence by biological reactions. The isolation of these compounds from the usual body liquids is a very difficult task and the quantity actually isolated is probably never more than a small per cent of that actually present. The large amounts of material necessary for the isolation of imidazoles present in small quantities make the process of isolation so tedious and time-consuming that it is hardly applicable to cases where a routine analysis is desired. The biological methods, based on the action of histamine on the smooth muscle fiber system, that have been used for the identification of the base, are certainly not quantitatively exact and have not been shown to be entirely specific. A purely chemical method, applicable to small amounts of material, would be superior to any that had been previously described, if the new method were accurate and practically applicable.

In the preceding communication a method for estimating small amounts of imidazoles has been described. In that paper no claims were made for the applicability of the method to mixtures of imidazoles. It is the object of this report to show that the method can also be applied to mixtures and that the exact quantity of histamine, histidine, and methyl imidazole in such mixtures can be rapidly and accurately determined. The method has been

found to be applicable without modification to liquid media in which bacteria have been allowed to metabolize histidine in the presence of salts and glycerol or glucose.[1] In its present form the method is not directly applicable, in its entirety, to more complex liquids such as urine or blood. We are trying to modify it, at the present time, so that it can be applied to such liquids. The underlying principles of the method are as follows.

The mixture of imidazole derivatives is treated with enough solid sodium hydroxide to give a 20 per cent solution of the alkali. This alkaline liquid is then extracted six times with twice its volume of amyl alcohol. This effects a separation into two fractions, the amyl alcohol extract and the alkaline aqueous liquid. The amyl alcohol extract contains all the histamine and methyl imidazole, together with ammonia and other amines if such are present in the original solution. We shall call this the *histamine fraction.* The alkaline aqueous liquid contains all the histidine, imidazole propionic, acetic, and lactic acids. We shall call this the *histidine fraction.*

The amount of histidine can then be determined by an amino nitrogen determination on the histidine fraction.

A color value determination on the histidine fraction is an index of the combined imidazole content of the liquid. If the color value determination indicates the presence of more imidazole than can be accounted for on the basis of the amino nitrogen determination, histidine is not the only imidazole present. The excess of color value can then be calculated to imidazole propionic, acetic, or lactic acids depending upon which of these substances is shown to be present by actual isolation.

Of all the imidazoles tried, methyl imidazole is the only one that is volatile with steam. It can be determined directly by collecting the distillates from a steam distillation, determining their color value, and calculating the amount of methyl imidazole from Table V of the previous article. It may be well to call attention here to the fact that imidazole itself is probably also volatile with, steam and if it is present, it will be estimated as methyl imidazole. No effort has been made to differentiate these two substances.

[1] Koessler, K. K., and Hanke, M. T., *J. Biol. Chem.*, 1919, xxxix, 539.

The histamine and methyl imidazole can be completely extracted from the amyl alcohol with 1.0 N H_2SO_4. A color value determination on the acid extract gives the total amount of imidazole present. By subtracting from this the color value due to the methyl imidazole, as previously determined, the color value due to histamine is obtained which can then be calculated to that substance by using Table II given in the previous paper. The bacterial experiments, described in the succeeding paper, showed methyl imidazole to be regularly absent. In this case, then, the color value determination gave the amount of histamine directly.

Sections I to VI of this report contain a detailed account of the results of experiments on known solutions of imidazole derivatives by which the accuracy of the technique of the method for separating histamine from histidine, described in Section VII, was experimentally established.

EXPERIMENTAL.[2]

I. Color Value of a Histidine-Histamine Mixture is the Sum of Their Individual Color Values.

(*a*) 1 cc. each of stock 1 per cent histidine and histamine dichloride solutions was mixed in a volumetric flask and diluted with water to exactly 100 cc.

Of this solution 0.10 cc. had a color value equivalent to 12.6 mm. (CR-MO).

This amount of each of the imidazoles alone would give

Histidine	5.0 mm. (CR-MO)
Histamine	7.5 " "
Theory	12.5 " "

(*b*) 1 cc. of the stock histamine solution and 0.50 cc. of the stock histidine solution were mixed in a volumetric flask and diluted with water to exactly 100 cc.

Of this solution 0.10 cc. had a color value equivalent to 10.0 mm. (CR-MO).

[2] The reagents and the terminology used are identical with those of the previous article (p. 497). The recorded readings have all been corrected by subtracting 0.30 mm., for the correction blank (p. 511).

This amount of each of the imidazoles alone would give

Histidine	2.5 mm. (CR-MO)
Histamine	7.5 " "
Theory	10.0 " "

Conclusion.—Histidine and histamine do not interfere with each other's color production. Each produces its color as if the other were absent.

II. Stability of Histidine and Histamine toward Acid and Alkali.

(*a*) *Cold 20 Per Cent Sodium Hydroxide.*—1 cc. each of the stock histidine and histamine solutions was mixed with 2 gm. of solid sodium hydroxide and enough water to give a total volume of 10 cc. After standing for 1 hour at room temperature the liquid was rendered faintly acid to litmus paper with 1.0 N HCl and diluted to exactly 100 cc.

Of this solution 0.10 cc. had a color value equivalent to 12.7 mm. (CR-MO).

The theory is 12.5 mm. (CR-MO) (see Section I) the result, therefore, is accurate within the limits of experimental error.

(*b*) *Hot 20 Per Cent Sodium Hydroxide.*—2 cc. of stock histidine solution were mixed with 8 cc. of water and 2 gm. of solid sodium hydroxide in a 100 cc. volumetric flask. The flask was heated on the boiling water bath for 7 hours. The cooled solution was neutralized as in (*a*) and diluted to 100 cc.

Of this solution 0.10 cc. had a color value equivalent to 8.4 mm. (CR-MO).

This, by table, is equal to 84 per cent of the histidine started with. The 7 hours heating with concentrated sodium hydroxide destroyed the imidazole ring to the extent of 16 per cent.

(*c*) An experiment was conducted on 2 cc. of stock histamine solution, the details of which were identical with those described under (*b*).

Of the final solution 0.10 cc. had a color value equivalent to 13.8 mm. (CR-MO).

This, by table, is equal to 92.5 per cent of the histamine started with. The 7 hours heating with concentrated alkali destroyed the imidazole ring to the extent of 7.5 per cent.

(*d*) *Hot Concentrated Hydrochloric Acid.*—2.00 cc. of stock histidine solution and 25 cc. of 37 per cent HCl were mixed in a 100 cc. lipped beaker and heated for 10 hours on the boiling water bath, rapid evaporation being prevented by covering the beaker with a watch-glass. At the end of the 10 hours heating the hydrochloric acid had disappeared. The dry residue was dissolved in water and diluted to exactly 100 cc.

Of this solution 0.10 cc. had a color value equivalent to 10.0 mm. (CR-MO).

This is the theoretical value.

(*e*) An experiment was conducted on 2 cc. of stock histamine solution, the details of which were identical with those described under (*d*).

0.10 cc. of the final solution had a color value equivalent to 15.0 mm. (CR-MO).

This is the theoretical value.

Conclusions.—Cold 20 per cent sodium hydroxide and hot concentrated hydrochloric acid do not change the color value of either histidine or histamine. Hot 20 per cent sodium hydroxide destroys 7.5 and 16 per cent of the color values of histamine and histidine respectively in the course of 7 hours. Since this loss in color value is probably caused by a destruction of the imidazole ring, it is clear that this heterocyclic ring is not perfectly stable toward hot alkali. The stability of the ring seems to depend to some extent upon the character of the side chain.

For quantitative results, a solution containing imidazoles may be evaporated to dryness with hydrochloric acid with impunity. Heating a strongly alkaline solution must be avoided.

III. Histidine Not Extracted from a 20 Per Cent Sodium Hydroxide Solution by Amyl Alcohol.

In order to prove that histidine is not extracted from a 20 per cent sodium hydroxide solution by amyl alcohol it was necessary to devise a method for extracting the alkaline solution with amyl alcohol that would be rapid and accurate. The following technique was finally adopted.

Extraction Tube and Mode of Extraction.—A 35 cc. glass-stoppered bottle was used as an extraction tube. The imidazole so-

lution, in this case 1.00 cc. of stock histidine solution, was introduced into the bottle together with enough water to give a total volume of about 10 cc. Then 2 gm. of Merck's highest purity sodium hydroxide were dissolved in the liquid, the bottle and con-

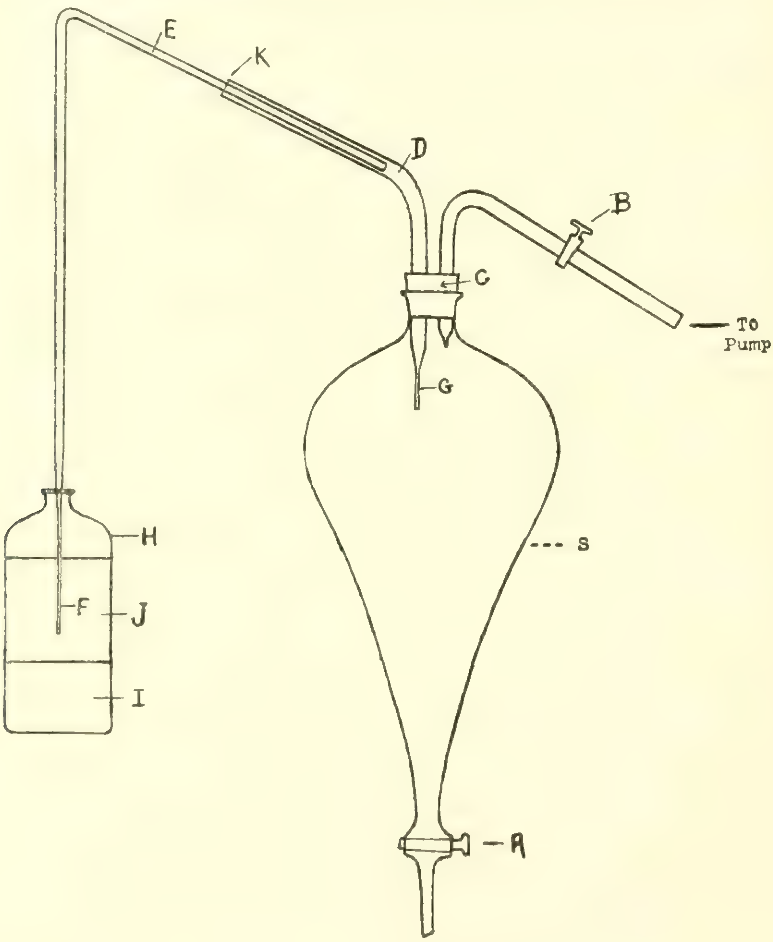

FIG. 1. A, Lower stop-cock of the Squibb separatory funnel S. B, Stopcock to outlet tube. C, Two-hole rubber stopper. D, Wide glass tube. E, Narrow glass tube. F, Capillary tube. G, Constriction in D, about 1 mm. diameter. H, Glass-stoppered extraction tube. I, Aqueous layer. J, Amyl alcohol layer. K, A piece of rubber tubing is used at this place to connect Tubes D and E.

tents being cooled in tap water while the alkali was dissolving. 20 cc. of prepared amyl alcohol,[3] measured by graduate, were introduced into the bottle, the glass stopper[4] inserted, and the liquids vigorously mixed for a few minutes. The bottle was then transferred to a centrifuge tube[5] and centrifuged for from 1 to 2 minutes. This gave a sharp separation into two layers.

Separation of Amyl Alcohol.—The amyl alcohol layer was separated from the aqueous alkaline layer by means of the device shown in Fig. 1.

The 300 cc. Squibb funnel S, which must be equipped with a tightly fitting glass stopper, and the extraction tube H are supported by means of two universal clamps which in turn are attached to the same ring-stand.

After the mixture in the extraction tube has been centrifuged into two sharply defined layers, the tube is clamped into place and raised until the capillary F is immersed in the amyl alcohol layer. Stop-cock B, which should be attached to an aspirator, is then opened very slightly so that a very slight drop in pressure occurs in the Squibb funnel. The amyl alcohol is sucked through tubes E and D into the funnel. By carefully raising the extraction tube H, the amyl alcohol can be almost entirely removed without disturbing the lower layer I. The extraction tube H is then lowered, tube E swung around so that capillary F points straight up and stop-cock B opened wide to sweep the amyl alcohol from tubes D and E into the funnel.

The alkaline aqueous layer is then ready to be extracted with another 20 cc. portion of amyl alcohol. The above process was repeated five times in this case, so that a total of 100 cc. of amyl alcohol was used.

[3] The amyl alcohol was prepared by distilling Merck's highest purity product *in vacuo* and extracting the distillate once with 20 per cent sodium hydroxide solution. This gives a product that is already saturated with water and sodium hydroxide with respect to a 20 per cent sodium hydroxide solution. The volume of the aqueous alkaline layer in the extraction tube will, therefore, remain constant.

[4] The glass stopper must fit perfectly throughout its entire length. It must not be removed from the bottle until after the liquid has been centrifuged.

[5] A deep layer of cotton makes an ideal cushion for the flat bottomed bottle.

Removal of Imidazoles from Amyl Alcohol.—The combined amyl alcohol extracts were extracted five times in the same Squibb funnel with 1.0 N H_2SO_4, using 20 cc. for the first and 10 cc. for each of the remaining four extracts. The sulfuric acid extracts were collected in a 100 cc. glass-stoppered precision cylinder and neutralized to litmus paper with 40 per cent sodium hydroxide. The solution was then rendered very faintly acid by adding a few drops of 1.0 N H_2SO_4, cooled to room temperature in tap water, and diluted to exactly 100 cc. with distilled water. About 20 cc. of chloroform were then added[6] and the mixture was vigorously agitated. The clear aqueous layer was used for the colorimetric determinations.

In this case 1.0 cc. had a color value equivalent to 0.20 mm. (CR-MO) which, by table, is equal to about 0.40 per cent of the histidine originally introduced.

The Alkaline Aqueous Liquid (*Histidine Fraction*).—The alkaline aqueous liquid was carefully transferred with water from the extraction tube to a 100 cc. precision cylinder, made slightly acid with hydrochloric acid, and, after cooling to room temperature, diluted to 100 cc. About 20 cc. of chloroform were then added[6] and the mixture was vigorously agitated. The clear aqueous layer was used for the colorimetric determinations.

In this case 0.10 cc. had a color value equivalent to 5.0 mm. (CR-MO); 0.20 cc. had a color value equivalent to 10.0 mm. (CR-MO), which, by table, is equivalent to 100 per cent of the histidine introduced.

Conclusion.—Histidine is extracted from a 20 per cent sodium hydroxide solution only to the extent of about 0.50 per cent by amyl alcohol under the prescribed conditions. This is negligible in most cases.

IV. Histamine Quantitatively Extracted from a 20 Per Cent Sodium Hydroxide Solution by Amyl Alcohol.

1 cc. of stock 1 per cent histamine solution was diluted to 10 cc. in the extraction tube. 2 gm. of solid sodium hydroxide were

[6] The chloroform removes the amyl alcohol from the aqueous layer completely. Amyl alcohol interferes actively with the colorimetric determinations; so it is necessary to take this precaution to be sure that the readings obtained are accurate.

then added and the alkaline aqueous liquid extracted six times with prepared amyl alcohol. The details of this experiment were duplicates of those described under Section III.

Alkaline Aqueous Liquid.—The solution finally obtained had absolutely no color value beyond that of the correction blank.

Amyl Alcohol Extract (*Histamine Fraction*).—Of the solution finally obtained, 0.10 cc. had a color value equivalent to 7.5 mm. (CR-MO); 0.20 cc. had a color value equivalent to 15.0 mm. (CR-MO). This, by table, is equivalent to 100 per cent of the histamine started with.

Conclusion.—Histamine is quantitatively extracted from a 20 per cent sodium hydroxide solution by amyl alcohol.

V. Histamine Quantitatively Separated from Histidine by Means of Amyl Alcohol.

(A) When Equal Parts by Weight of Histidine and Histamine Are Present.

1 cc. each of stock histidine and histamine solutions was diluted to 10 cc. in the extraction tube. 2 gm. of solid sodium hydroxide were then added and the alkaline aqueous liquid extracted five times with prepared amyl alcohol. The details of this experiment were duplicates of those described under Section III.

Alkaline Aqueous Liquid (*Histidine Fraction*).—Of the solution finally obtained, 0.20 cc. had a color value equivalent to 10.2 mm. (CR-MO). This is equivalent to 0.0102 gm. of histidine dichloride which is 102 per cent of the histidine originally introduced.

Amyl Alcohol Extract (*Histamine Fraction*).—Of the solution finally obtained, 0.10 cc. had a color value equivalent to 7.4 mm. (CR-MO); 0.20 cc. had a color value equivalent to 14.7 mm. (CR-MO). This is equivalent to 0.0098 gm. of histamine dichloride which is 98 per cent of the starting material.

Discussion.—In this case both results are accurate to within the limit of experimental error; but we are now certain that the low value for the histamine fraction was obtained because too few amyl alcohol extracts were made. In the following two experiments where six extracts were made instead of five, the agreement

was perfect. It is necessary, therefore, to make not less than six amyl alcohol extracts to effect a quantitative separation of histamine from histidine.

(B) When a Small Amount of Histamine Is Mixed with a Large Amount of Histidine.

5 cc. of stock histidine solution and 0.30 cc. of stock histamine solution were diluted to 10 cc. in the extraction tube. 2 gm. of solid sodium hydroxide were then added and the alkaline aqueous liquid extracted six times with prepared amyl alcohol. The details of this experiment were duplicates of those described under Section III.

Histidine Fraction.—As the solution finally obtained was too concentrated to make an accurate determination possible 10 cc. of it were diluted to 100 cc. Of this diluted solution, 0.40 cc. had a color value equivalent to 10.0 mm. (CR-MO). This is equivalent to 0.050 gm. of histidine dichloride for the entire original solution which is a 100 per cent recovery.

Histamine Fraction.—Of the solution finally obtained, 0.10 cc. had a color value equivalent to 22.7 mm. (CR-MO). This is equivalent to 0.003025 gm. of histamine dichloride for the entire solution which is 100.9 per cent of the starting material.

(C) When a Large Amount of Histamine is Mixed with a Small Amount of Histidine.

4.5 cc. of stock histamine solution and 0.50 cc. of stock histidine solution were diluted to 10 cc. in the extraction tube. 2 gm. of solid sodium hydroxide were then added and the alkaline aqueous liquid extracted six times with prepared amyl alcohol. The details of this experiment were duplicates of those described under Section III.

Histidine Fraction.—Of the solution finally obtained, 0.40 cc. had a color value equivalent to 10.0 mm. (CR-MO). This is equivalent to 0.005 gm. of histidine dichloride for the entire solution which is 100 per cent of the amount originally introduced.

Histamine Fraction.—As the solution finally obtained was too concentrated to make an accurate determination possible 10 cc. of it were diluted to 100 cc.

Of this diluted solution 0.40 cc. had a color value equivalent to 13.5 mm. (CR-MO). This is equivalent to 0.045 gm. of histamine dichloride for the entire solution which is 100 per cent of the amount originally introduced.

Conclusion.—Histamine can be quantitatively separated from histidine regardless of the proportion in which each is present.

VI. Estimation of Methyl Imidazole.

(A) Methyl Imidazole Slightly Volatile with Steam from Alkaline Solution[7] *(Method A).*

1 cc. of stock 1 per cent methyl imidazole was diluted to 100 cc. in a glass dish, treated with 5 cc. of a M Na_2CO_3 solution, and evaporated on the boiling water bath until the final volume of the alkaline liquid was about 5 cc. An excess of hydrochloric acid was then carefully added and the solution evaporated until all the water and acid had volatilized. The residue was transferred, with water, to a 100 cc. volumetric flask and diluted to exactly 100 cc.

Of this solution 0.10 cc. had a color value equivalent to 20.2 mm. (CR), which is equivalent to 0.0075 gm. of methyl imidazole or 75 per cent of the amount originally introduced. This treatment will volatilize 0.0025 gm. of methyl imidazole; so if less than this amount is present in the solution it should be determinable by this method which we shall call Method A. The mode of applying this method to a practical unknown is given in Section VII.

(B) Methyl Imidazole Volatilizes Slightly from a Solution Containing Hydrochloric Acid.[7]

1 cc. of stock methyl imidazole was diluted with water to about 100 cc. in a glass dish. Then 5 cc. of 37 per cent HCl were added and the solution evaporated on the water bath until all the water and acid had been volatilized. The residue was transferred to a volumetric flask and diluted with water to 100 cc.

[7] These two experiments, (*A*) and (*B*), do not prove that the methyl imidazole was actually lost by volatilization. That this imidazole derivative is really volatile with steam was conclusively demonstrated and the proof of this statement is given under Method B.

Of the solution so obtained 0.10 cc. had a color value equivalent to 23.7 mm. (CR), which is equivalent to 0.00875 gm. of methyl imidazole or 87.5 per cent of the amount originally introduced. It appears, then, that 0.00125 gm. of methyl imidazole volatilized even in the presence of hydrochloric acid. This may mean either that methyl imidazole hydrochloride is hydrolyzed by water and that the base formed by hydrolysis is volatilized, or that methyl imidazole hydrochloride is itself volatile. The following experiment has convinced us that it is the hydrochloride that is volatile.

These experiments are of value because they show clearly that a solution in which methyl imidazole is to be determined must not have been evaporated in the presence of either alkali or hydrochloric acid.

(C) Methyl Imidazole Will Not Volatilize from a Solution Containing Sulfuric Acid.

1 cc. of stock methyl imidazole solution was diluted with 100 cc. of water in a glass dish. Then 1 cc. of 95 per cent H_2SO_4 was added to the solution and the liquid evaporated to a final volume of about 5 cc. This was transferred to a volumetric flask, neutralized to litmus paper, and diluted to 100 cc.

Of this solution 0.10 cc. had a color value equivalent to 27.0 mm. (CR), which is equivalent to 100 per cent of the amount introduced.

When it is necessary to concentrate a solution before determining its color value, this can be safely done after adding an excess of sulfuric acid.

(D) Direct Determination of Methyl Imidazole (Method B).

5 gm. of anhydrous sodium carbonate, 5 cc. of water, and 1 cc. of stock methyl imidazole solution were subjected to a steam distillation from a 200 cc. distilling flask, the volume of the methyl imidazole-containing solution being kept constantly at from 5 to 10 cc.[8] The distillate was collected in a 250 cc. graduated precision cylinder.

[8] The liquid from which the methyl imidazole is to be volatilized must be kept saturated with sodium carbonate. Methyl imidazole is practically non-volatile from a dilute sodium carbonate solution.

Collections.—The volume of the first distillate was 154 cc., of which 0.10 cc. had a color value equivalent to 14.8 mm. (CR). The entire 154 cc. contained 0.00847 gm. of methyl imidazole.

The volume of the second distillate was 153 cc. of which 0.50 cc. had a color value equivalent to 8.7 mm. (CR). The entire 153 cc. contained 0.0009945 gm. of methyl imidazole.

The volume of the third distillate was 150 cc. of which 1.0 cc. had a color value equivalent to 6.0 mm. (CR). The entire 150 cc. contained 0.000338 gm. of methyl imidazole.

The volume of the fourth distillate was 140 cc. of which 1.0 cc. had a color value equivalent to 1.10 mm. (CR). The entire 140 cc. contained 0.00007 gm. of methyl imidazole.

There was thus accounted for 0.0098725 gm. of methyl imidazole which is 98.7 per cent of the amount originally introduced.

(E) Proof That Methyl Imidazole Can Be Estimated by Method B in the Presence of Other Imidazoles.

1 cc. each of the stock histidine, histamine, methyl imidazole, imidazole propionic acid, and imidazole acetic acid solutions was mixed and subjected to steam distillation under conditions identical with those given above. Six distillates were collected, the combined content of which was 0.009831 gm. of methyl imidazole which is 98.31 per cent of the methyl imidazole originally introduced. This proves conclusively that of the five imidazoles tried, only methyl imidazole is volatile with steam, and that it can be separated from other imidazoles by this method. Since methyl imidazole is chemically similar to *p*-cresol and scatole, both of which have been found in animal excretions, it will be of interest to see if methyl imidazole cannot also be found in such excretions. We have been unable to establish this as a fact up to the present time.

(F) Method C for Estimating Methyl Imidazole.

Method A has proved to be untrustworthy when applied to solutions containing the bacterial decomposition products of glycerol because evaporation with alkali gives rise to non-volatile substances that interfere with the colorimetric determinations. The amyl alcohol extract from such solutions is free from these decomposition

products. It should be possible, therefore, to determine methyl imidazole by evaporating the *histamine fraction* with alkali as described under Method A, instead of the *original solution*. In this case a color value determination on the histamine fraction would give the total amount of imidazole present. The solution would then be evaporated to dryness with 5 cc. of M Na_2CO_3 solution, the residue dissolved in hydrochloric acid and the solution reevaporated. The residue would be dissolved in water and diluted to the original volume of the histamine fraction. The color value so obtained would be *lower* than that of the original histamine fraction only when methyl imidazole was originally present and the decrease in color value could easily be calculated to methyl imidazole. For bacterial metabolism studies this method has been found to be quite accurate and shorter than Method B. Its application is more specifically given in Section VII.

VII. Separation of Histamine and Methyl Imidazole from Histidine, Imidazole Propionic Acid, and Imidazole Acetic Acid. The Accurate Determination of Histamine, Methyl Imidazole, and Histidine.

The method outlined below is a duplicate of the method that was used in the bacterial metabolism studies to be described in the following paper. Some of the steps were taken, not because they were necessary in this case, but because they were necessary in the metabolism experiments.

A solution containing the following was prepared from the stock solutions: 5 cc. of 2 per cent histidine dichloride, 4 cc. of 1 per cent histamine dichloride, 2 cc. of 1 per cent imidazole propionic acid, 2 cc. of 1 per cent imidazole acetic acid, and 2 cc. of 1 per cent methyl imidazole. This was mixed with 10 cc. of 2 per cent ammonium chloride, 4 cc. of glycerol, 100 cc. of Salt Medium 1[1], and 76 cc. of water in a 300 cc. Pyrex Florence flask.

Hydrogen Ion Concentration.—1 cc. of the finished solution was treated with 0.10 cc. of a 0.01 per cent solution of phenolsulfonephthalein and the color produced compared with a set of standard tubes.[9] In this way the original solution was found to have a pH of 6.8.

[9] Furnished by Hynson, Westcott, and Dunning, Baltimore, Md.

Filtration.—The remaining solution was forced through a Berkefeld filter. The flask and filter were washed free from imidazoles with 200 cc. of distilled water. The filtrate was treated with 1 cc. of 95 per cent sulfuric acid, transferred to a 200 cc. glass dish and evaporated on the water bath until salts began to crystallize. The residue was carefully transferred to a graduated precision cylinder with enough water to give a final volume of exactly 25 cc. This will be referred to as the test solution.

Total Color Value.—2.50 cc. of the test solution were diluted to 100 cc. in a volumetric flask. Of this solution 0.10 cc. had a color value equivalent to 17.7–17.9 mm. (CR-MO) the color being slightly too red for the standard indicator. 0.10 cc. of this solution should contain

	gm.		*mm.*	
Histidine dichloride	0.00001	Color value	5.0	(CR-MO)
Histamine "	0.000004	" "	3.0	"
Methyl imidazole	0.000002	" "	1.9	"
Imidazole propionic acid	0.000002	" "	2.7	"
" acetic "	0.000002	" "	2.3	"
Theory			17.9	"

The check between fact and theory is perfect which adds weight to the statement made in Section I. That so close a check should have been obtained is surprising when the different rates of color development for these five substances are considered. The results obtained here show that *imidazoles are not adsorbed by a Berkefeld filter.*

Methyl Imidazole (*Method A*).—The remaining 98 cc. of the above solution were transferred to a glass dish, treated with 5 cc. of a M Na_2CO_3 solution, and evaporated to a volume of about 5 cc. 50 cc. of water were then added and the solution reevaporated to a volume of 5 cc. Finally 5 cc. of 37 per cent HCl were added carefully and the liquid was evaporated until all the water and acid had volatilized. The residue was dissolved in water and diluted to exactly 98 cc.

Of this solution 0.10 cc. had a color value equivalent to 13.2 mm. (CR-MO). 17.9 − 13.2 = 4.7 mm. (CR-MO) or 5.2 mm. (CR)[10] was the color value due to methyl imidazole. This, by

[10] Koessler, K. K., and Hanke, M. T., *J. Biol. Chem.*, 1919, xxxix, 497.

table, is equivalent to 0.0193 gm. of methyl imidazole for the entire test solution which is 97 per cent of that actually introduced.

Separation of the Histamine from the Histidine Fraction.—10 cc. of the test solution were measured into the extraction tube. 3 gm. of solid sodium hydroxide were added to the solution and the mixture cooled in tap water while the alkali was dissolving. The strongly alkaline aqueous liquid was extracted six times with prepared amyl alcohol in the manner described in Section III.

Histidine Fraction.—The alkaline liquid was carefully transferred to a graduated precision cylinder with 10 cc. of 20 per cent N HCl. The liquid, which was still alkaline to litmus paper, was rendered faintly acid by adding 95 per cent H_2SO_4 drop by drop. Water was then added to the cooled solution to bring its volume up to exactly 25 cc. About 10 cc. of chloroform were finally added[6] and the mixture was vigorously agitated.

Color Value.—6.25 cc. of the clear aqueous layer were diluted to 100 cc. in a precision cylinder and vigorously agitated with about 20 cc. of chloroform. Of the clear aqueous layer, 0.10 cc. had a color value equivalent to 11.0 mm. (CR). In this case the (CR) indicator gave a better match than the (CR-MO) indicator. 11.0 mm. (CR) are equivalent to 10.0 mm. (CR-MO).[10] The color value of this solution should have been

	gm.		*mm.*	
Histidine dichloride...........	0.00001	Color value	5.0	(CR-MO)
Imidazole propionic acid......	0.000002	" "	2.7	"
" acetic "	0.000002	" "	2.3	"
Theory..			10.0	"

The agreement was perfect. This proves that histidine, imidazole propionic acid, and imidazole acetic acid are quantitatively retained by the alkali and appear together in the histidine fraction. Of these three substances only one, histidine, can be determined directly. We have been unable to devise a method for estimating either imidazole acetic or propionic acid separately, so that if both should appear in an unknown it would be impossible to differentiate between them. Their combined quantity can be determined by subtracting from the total that part of the color value that is due to histidine, as calculated from the Van Slyke determination, and calculating the excess color value to either imidazole propionic or acetic acid.

Amino Nitrogen Determination (*Histidine*).—The amino nitrogen in 5 cc. of the histidine fraction was determined by the Van Slyke method using the micro-apparatus. There was thus obtained 0.88 cc. of N_2 at 16° and 749 mm., which is equivalent to 0.1024 gm. of histidine dichloride for the entire test solution. This is 102.4 per cent of the amount originally introduced which is within the limit of experimental error for so small a quantity of substance. This proves that the ammonia present in the original test solution has passed into the amyl alcohol extract so that no special precautions need be taken to free the histidine fraction from ammonia. This statement has been amply verified by experiments described in the following paper. It need hardly be said that this method will be an accurate index of the quantity of histidine in the test solution only when other amino-acids are absent.

The Histamine Fraction.—The combined amyl alcohol extracts were extracted with 1.0 N H_2SO_4 as has already been described under Section III. The solution finally obtained was too concentrated to make an accurate determination possible, so 12.5 cc. of it were diluted to 50 cc. in a volumetric flask.

Of this diluted solution 0.10 cc. had a color value equivalent to 8.7 mm. (CR) or 7.9 mm. (CR-MO) and 0.20 cc. had a color value equivalent to 17.4 mm. (CR) or 15.8 mm. (CR-MO).

If the methyl imidazole and histamine had been entirely extracted by the amyl alcohol, 0.10 cc. of this solution should have had a color value equivalent to 7.9 (CR-MO). The check is perfect.

Removal of Ammonia and Methyl Imidazole by Evaporation with Alkali.—The entire histamine fraction was recombined and evaporated in a glass dish with 5 cc. of a M Na_2CO_3 solution until a dry residue was obtained. This was then dissolved in water, treated with an excess of 37 per cent HCl, and the resulting solution again evaporated to remove the water and acid entirely. The solid so obtained was transferred to a precision cylinder with enough water to give a final volume of 20 cc.

Amino Nitrogen Determination (*Histamine*).—Of the above solution, 5 cc. gave 0.56 cc. of N_2 at 25° and 747 mm., which is equivalent to 0.04015 gm. of histamine dichloride for the entire test solution and is 100.4 per cent of that actually introduced. Although this method works ideally when pure substances are

used, it occasionally gives high results when applied to bacterial metabolism experiments because this fraction sometimes contains other non-volatile amines.

Color Value Determination (*Methyl Imidazole by Method C*).—5 cc. of the concentrated histamine solution were diluted to 100 cc. in a volumetric flask. Of this diluted solution 0.20 cc. had a color value equivalent to 6.0 mm. (CR-MO) which is equivalent to 0.04 gm. of histamine dichloride for the entire test solution and 100 per cent of the amount originally introduced. The evaporation with the alkali produced a loss in color value of $15.8 - 6.0 = 9.8$ mm. (CR-MO) or 10.8 mm. (CR) for 0.20 cc. of the solution which, by table, is equivalent to 0.02 gm. of methyl imidazole for the entire test solution and is 100 per cent of the amount originally introduced.

This method gives the exact quantities of both histamine and methyl imidazole. As will be shown in the following paper, equally exact values are obtained when bacterial metabolism liquids are examined.

Determination of Ammonia.

The method of Van Slyke and Cullen[11] was used for the determination of ammonia. 0.10 N HCl and NaOH were used in place of the 0.02 N acid and alkali recommended by the above authors. 5 gm. of solid potassium carbonate were added to 5 cc. of the test solution and the mixture aerated for 1 hour. The liberated ammonia neutralized 7.25 cc. of the 0.1 N acid as compared to 7.28 cc. demanded by the theory for 97.4 per cent ammonium chloride which was the purity of the salt originally introduced.

CONCLUSION.

A method has been devised for separating histamine and methyl imidazole from histidine, and imidazole propionic and imidazole acetic acids.

To Professor F. C. Koch, acting Chairman of the Department of Physiological Chemistry, we are indebted for suggestions and advice, in various ways, during the course of this work. We wish, herewith, to acknowledge his aid and to express our thanks for his many kindnesses.

[11] Van Slyke, D. D., and Cullen, G. E., *J. Biol. Chem.*, 1914, xix, 218.

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STUDIES ON PROTEINOGENOUS AMINES.

IV. THE PRODUCTION OF HISTAMINE FROM HISTIDINE BY BACILLUS COLI COMMUNIS.

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INTRODUCTION.

β-imidazolylethylamine (4-β-aminoethylglyoxaline), or histamine as it is appropriately called, was first obtained by Windaus and Vogt[1] in 1907 through synthesis from imidazole propionic acid. As a product of putrefaction of histidine it was first isolated by Ackermann[2] in 1910, who inoculated a synthetic medium containing histidine hydrochloride, peptone, glucose, and the necessary mineral salts with a piece of beef pancreas which had been allowed to putrefy for 24 hours. Aside from histamine, Ackermann was also able to separate a small quantity of imidazole propionic acid from the putrefaction mixture. In the same year the remarkale physiological properties of histamine were discovered independently by Barger and Dale,[3] and by Kutscher,[4] who found that it was one of the active principles of ergot. The physiological action of the base was then studied in great detail by many investigators, especially by Dale[5] and his coworkers In 1911 Barger and Dale[6] obtained histamine from the mucosa of the small intestine of the ox and ascribed the depressor action of secretin, at least in part, to this base. The question whether histamine is a normal product of the intestinal mucosa or whether it is found there as a product of the bacterial decomposition of proteins containing histidine was decided in favor of the latter assumption by Mellanby and Twort[7] who isolated, from

[1] Windaus, A., and Vogt, W., *Ber. chem. Ges.*, 1907, xl, 3691.

[2] Ackermann, D., *Z. physiol.. Chem.*, 1910, lxv, 504.

[3] Barger, G., and Dale, H. H., *Proc. Physiol. Soc.*, 1910, xxvi, 128; *Zentr. Physiol.*, 1910–11, xxiv, 885; *J. Chem. Soc.*, 1910–11, xcvii, 2592.

[4] Kutscher, F., *Centr. Physiol.*, 1910–11, xxiv, 163.

[5] Dale, H. H., and Laidlaw, P. P., *J. Physiol.*, 1910–11, xli, 318; 1911–12, xliii, 182.

[6] Barger, G., and Dale, H. H., *J. Physiol.*, 1910–11, xli, 499.

[7] Mellanby, E., and Twort, F. W., *J. Physiol.*, 1912–13, xlv, 53.

the intestinal contents of the guinea pig, a bacillus of the colon-typhoid group capable of converting histidine into histamine. In the same year Berthelot and Bertrand[8] isolated a bacillus from the human intestine that resembled Friedländer's pneumobacillus in all its morphological and chemical characteristics, but which had the additional faculty of decarboxylating histidine to histamine. They named this organism *Bacillus aminophilus intestinalis*.

This apparently simple chemical process, the decarboxylation of amino-acids to exceedingly potent substances of amine structure, is of great theoretical and practical interest. The relation of this problem to the general nutrition of bacteria, to the metabolism of amino-acids in the mammalian organism, to the pathology and pharmacology of the smooth muscle fiber system, and to the chemical constitution of the products of the glands of internal secretion, mark it as a fundamental inquiry of biology. The systematic investigation of these problems has been under way in this laboratory for the past 4 years.

Theoretical considerations and preliminary experiments had convinced us that the ability to decarboxylate amino-acids to amines could not be restricted to one or two species of bacteria but might well be a fairly common property of microorganisms. It soon became apparent that to attack the problem successfully it would be necessary to devise quantitative chemical methods for the determination and separation of the various proteinogenous amines as well as the other possible products resulting from the catabolism of the amino-acids. The preparation of the amino-acids, amines, intermediates, and by-products was an obvious preliminary to the attack on the bacterial metabolism experiments. We have already reported in detail on the preparation of β-imidazolylethylamine.[9] The preceding two articles in this *Journal* describe the quantitative methods used for the separation and estimation of imidazoles. It is the object of this communication to show what the optimum conditions are for the conversion of histidine to histamine by the colon bacillus. It is our intention to report a series of experiments in the near future that will show which of the common pathogenic and non-patho-

[8] Berthelot, A., and Bertrand, D.-M., *Compt. rend. Acad.*, 1912, cliv, 1643, 1826.

[9] Koessler, K. K., and Hanke, M. T., *J. Am. Chem. Soc.*, 1918, xl, 1716.

genic bacteria possesses the faculty of decarboxylating histidine. Work is also under way to apply similar methods to a study of the decarboxylation of tyrosine and tryptophane.

EXPERIMENTAL.

Foreword.

We have divided the experimental material presented in this article into three parts.

Part I contains a description of seventeen experiments that show the effect of *the composition of the medium* upon the metabolism of histidine by the colon bacillus.

The experiments described in Part II were carried out to ascertain *how the amount of histamine produced varied with the duration of the experiment.*

Part III contains a description of four experiments that seem to throw some light on *the causal relation between the formation of histamine and the hydrogen ion concentration of the medium.*

Part I. Effect of the Composition of the Medium upon the Metabolism of Histidine by the Colon Bacillus.

Preparation of Stock Solutions.

Stock Histidine Solution.—Vacuum-dried, 100 per cent histidine dichloride—4.0000 gm.—was dissolved in water and diluted to exactly 200 cc. in a volumetric flask.

Stock Ammonium Chloride Solution.—J. T. Baker's 97.4 per cent ammonium chloride—2.0000 gm.—was dissolved in water and diluted to exactly 100 cc.

Stock Potassium Nitrate Solution.—Kahlbaum's 100 per cent potassium nitrate—2.0000 gm.—was dissolved in water and diluted to exactly 100 cc.

Salt Medium 1.—0.30 gm. of potassium chloride.

8.00	"	of potassium dihydrogen phosphate.
24.00	"	of sodium chloride.
0.40	"	" sulfate (anhydrous).
8.00	"	" bicarbonate.
0.20	"	calcium chloride (anhydrous).

These amounts were dissolved in water and diluted to exactly 2,000 cc. When properly prepared the salt medium should contain no precipitate.

Mode of Preparing Completed Media.

The most complex media were Nos. 15 and 16. They were prepared as follows:

10 cc. each of the stock histidine and ammonium chloride solutions and 5 cc. of stock potassium nitrate solution were measured, by means of normal pipettes, into a 300 cc. Pyrex Florence flask. 4 cc. of glycerol, measured by graduate, were washed into the Pyrex flask with 71 cc. of water. Exactly 100 cc. of salt medium were then added.

Each of the seventeen completed media had a final volume of 200 cc. before sterilization, and contained exactly 0.2000 gm. of histidine dichloride.

Nos. 1 and 2 contained	10 cc. of stock 2 per cent histidine dichloride,
	100 " " salt medium,
	90 " " water.
Nos. 3 and 4 contained	10 cc. of stock 2 per cent histidine dichloride,
	10 " " " 2 " " ammonium choride,
	100 " " salt medium,
	80 " " water.
Nos. 5 and 6 contained	10 cc. of stock 2 per cent histidine dichloride,
	4 " " glycerol,
	100 " " salt medium,
	86 " " water.
Nos. 7 and 8 contained	10 cc. of stock 2 per cent histidine dichloride,
	10 " " " 2 " " ammonium chloride,
	4 " " glycerol,
	100 " " salt medium,
	76 " " water.
Nos. 9 and 10 contained	10 " " stock 2 per cent histidine dichloride,
	5 " " " 2 " " potassium nitrate,
	100 " " salt medium,
	85 " " water.
Nos. 11 and 12 contained	10 cc. of stock 2 per cent histidine dichloride,
	5 " " " 2 " " potassium nitrate,
	10 " " " 2 " " ammonium chloride,
	100 " " salt medium,
	75 " " water.
Nos. 13 and 14 contained	10 cc. of stock 2 per cent histidine dichloride,
	5 " " " 2 " " potassium nitrate,
	4 " " glycerol,
	100 " " salt medium,
	81 " " water.

Nos. 15 and 16 contained 10 cc. of stock 2 per cent histidine dichloride,
5 " " " 2 " " potassium nitrate,
10 " " " 2 " " ammonium chloride,
4 " " glycerol,
100 " " salt medium,
71 " " water.

No. 17 contained 10 cc. of stock 2 per cent histidine dichloride,
5 " " " 2 " " potassium nitrate,
10 " " " 2 " " ammonium chloride,
5 gm. of crystalline glucose,
100 cc. of Salt Medium 1,
70 " " distilled water.

Sterilization.

The seventeen flasks were stoppered with cotton and sterilized by autoclaving for 1 hour at 10 pounds pressure. Calcium phosphate precipitated as carbon dioxide escaped.

Hydrogen Ion Concentration before Inoculation.

This was determined by the method described on page 534. Each of the sixteen completed media had a pH of 7.35 before inoculation.

Inoculation with Bacillus coli communis.

The culture used was a laboratory stock culture, originally isolated from human feces.

The 24 hours growth on sixteen agar slants was transferred with sterile salt solution to a sterile 25 cc. graduated precision cylinder. The emulsion was then diluted to 25 cc. with sterile salt solution.

Method for Counting Bacteria.

Of the well mixed emulsion, 1 cc. was diluted to 250 cc. Of this dilute suspension, 0.01 cc. was spread out smoothly over an area of 10 sq. cm. and the bacteria stained with 5 per cent aqueous fuchsin. They were then counted by the method of Breed and Brew[10] which showed that about 9,000,000,000 bacteria were

[10] Breed, R. S., and Brew, J. D., *New York Agric. Exp. Sta., Tech. Bull. 49*, 1916.

present in every cc. of the original bacterial emulsion. Exactly 1 cc. of the original emulsion was transferred to each of the sixteen flasks.

Incubation.

The nine odd numbered flasks were placed in an incubator. This is the aerobic series. The eight even numbered flasks were placed in a large vacuum desiccator with a dish containing pyrogallic acid and sodium hydroxide. The desiccator was completely exhausted, placed in the incubator, and reexhausted once a day to insure against leakage which was very slight. This is the anaerobic series.

The mixtures were incubated for 14 days.

Growth of Bacteria.

Judging from the degree of turbidity of the solutions, the bacteria grew best in Flasks 7, 8, 13, 14, 15, and 16. A very good growth was also obtained in Flasks 5, 6, 11, and 17. Flasks 12, 9, 10, 3, 4, 1, and 2 had the least growth in descending order.

The aerobic solutions were pale yellow to green, the anaerobic were colorless to very pale yellow.

Filtration.

The contents of each flask were forced through a clean 2.5 inch Berkefeld filter. The flask and filter were then washed free from imidazoles with about 200 cc. of distilled water. The filtrate obtained was always perfectly clear.

Hydrogen Ion Concentration after Incubation and Filtration.

1 cc. of the undiluted filtrate was used for the pH determination. The following results were obtained.

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
pH	*pH*	*pH*	*pH*	*pH*	*pH*	*pH*	*pH*	*pH*	*pH*	*pH*	*pH*	*pH*	*pH*	*pH*	*pH*	*pH*
8.3	7.6	8.2	7.4	7.15	7.45	>6.6	>6.6	8.4	8.0	8.3	7.9	>6.6	>6.6	>6.6	>6.6	>6.6

Test Solution.

The filtrate and washings were treated with 1 cc. of 95 per cent H_2SO_4 and evaporated in a 200 cc. glass dish until salts began to crystallize. This residue was then transferred to a precision cylinder with enough water to give a final volume of 25 cc. This liquid will be referred to as the test solution.

Total Color Value.

2.50 cc. of the test solution were diluted to exactly 100 cc. Portions of this diluted solution were then used for the colorimetric determinations. The results obtained are given in Table I.

TABLE I.

Total Color Value.

Flask No.	Diluted solution.	Color value of (CR-MO) indicator.		Relation of total color value to the original color value.
	cc.	*mm.*		*per cent*
1	1.0	2.0	Brown, match poor.	2.0
2	0.10	6.6		66.0
	0.20	13.2		
3	1.0	3.5	Brown, match poor.	3.5
4	0.10	10.1		98.0
	0.20	20.3		
5	0.10	9.0		90.0
	0.20	18.0		
6	0.10	10.4		104.0
	0.20	20.8		
7	0.10	9.4		94.0
	0.20	18.8		
8	0.10	10.0		100.0
	0.20	20.0		
9	1.0	3.7	Brown.	3.7

TABLE I.—*Concluded.*

Flask No.	Diluted solution.	Color value of CR MO indicator.		Relation of total color value to the original color value.
	cc.	*mm.*		*per cent*
10	0.10	9.1		91.0
	0.20	18.2		
11	1.0	2.7	Brown.	2.7
12	0.10	10.0		100.0
	0.20	20.0		
13	0.10	10.0		100.0
	0.20	20.0		
14	0.10	9.7		97.0
	0.20	19.4		
15	0.10	10.0		100.0
	0.20	20.0		
16	0.10	10.0		100.0
	0.20	20.0		
17	0.10	9.3		93.0
	0.20	16.0		

Separation of the Histamine from the Histidine Fraction.

This separation was conducted on 10 cc. of the test solution exactly as has already been described on page 534. No separation was conducted on Flasks 1, 3, 9, and 11, because the total color value determination showed that the imidazole ring had been almost completely destroyed in these cases.

Histidine Fraction.—Of the histidine fraction, whose final volume was 25 cc., 6.25 cc. were diluted to 100 cc. for the color value determination, and 5 cc. portions were analyzed for amino nitrogen, all the steps being duplicates of the process already described. The results are given in Table II.

TABLE II.

Histidine Fraction.

Flask No.	Solution used.	Color value of (CR-MO) indicator.	Color value of histidine fraction as histidine.	Introduced histidine recovered unchanged (Van Slyke method) from 5 cc. of test solution.	Histidine converted into imidazole propionic acid (by difference).
	cc.	*mm.*	*per cent*		*per cent*
2	0.10 0.20	6.4 12.7	64	1.13 cc. N_2 at 20° and 748 mm. 0.1290 gm. histidine Cl_2. 64.5 %	0
4	0.10 0.20	10.1 19.8	98	1.80 cc. N_2 at 22.5° and 741 mm. 0.2008 gm. histidine Cl_2. 100.4 %	0
5	0.10 0.20	7.9 15.8	79	1.42 cc. N_2 at 20° and 752 mm. 0.1629 gm. histidine Cl_2. 81.5 %	0
6	0.10 0.20	7.4 14.8	74	1.05 cc. N_2 at 25° and 745 mm. 0.1162 gm. histidine Cl_2. 58.1 %	8.15
7	0.10 0.20	4.6 9.2	46	0.80 cc. N_2 at 20° and 754 mm. 0.092 gm. histidine Cl_2. 46.0 %	0
8	0.10 0.20	6.4 12.7	64	1.18 cc. N_2 at 24° and 742 mm. 0.131 gm. histidine Cl_2. 65.5 %	0
10	0.10 0.20	8.9 17.8	89	1.60 cc. N_2 at 25° and 753 mm. 0.1792 gm. histidine Cl_2. 89.6 %	0
12	0.10 0.20	9.8 19.7	98	1.75 cc. N_2 at 20° and 750 mm. 0.20 gm. histidine Cl_2. 100.0 %	0
13	0.10 0.20	4.0 8.0	40	0.70 cc. N_2 at 23° and 753 mm. 0.0791 gm. histidine Cl_2. 39.5 %	0

TABLE II—*Concluded.*

Flask No.	Solution used.	Color value (CR-MO) indicator.	Color value of histidine fraction as histidine.	Introduced histidine recovered unchanged (Van Slyke method) from 5 cc. of test solution.	Histidine converted into imidazole propionic acid (by difference).
	cc.	*mm.*	*per cent*		*per cent*
14	0.10	9.0	90	1.60 cc. N_2 at 23° and 753 mm.	0
	0.20	18.1		0.1807 gm. histidine Cl_2.	
				90.4 %	
15	0.10	4.5	45	0.85 cc. N_2 at 23° and 754 mm.	0
	0.20	9.0		0.0961 gm. histidine Cl_2.	
				48.0 %	
16	0.10	7.5	75	1.35 cc. N_2 at 20° and 753 mm.	0
	0.20	15.0		0.1549 gm. histidine Cl_2.	
				77.4 %	
17	0.10	5.3	53	1.06 cc. N_2 at 24° and 737 mm.	0
	0.20	8.2		0.1168 gm. histidine Cl_2.	
				58.4 %	

Discussion.—The colors produced by the histidine fraction were indistinguishable from those produced by pure histidine excepting No. 6. In this case the color produced was slightly too pink for the indicator which would suggest the presence of imidazole propionic, acetic, or lactic acid. The color value of No. 6 was equivalent to 74 per cent of the original color value. The Van Slyke determination showed that only 58 per cent of the histidine originally introduced was still present. It was quite clear, therefore, that in this one case a part of the histidine had been deaminized. We did not determine the character of the product but assumed it to be imidazole propionic acid because this substance has actually been isolated by Ackermann[2] as a product of the bacterial metabolism of histidine. If 58 per cent of the original color value is assumed to be due to histidine, this would be equivalent to 5.8 mm. (CR-MO) for 0.10 cc. of the solution. The difference between this and the observed color value—1.6 mm. (CR-MO)—would be due to imidazole propionic acid. This is equivalent to 0.01 gm. of imidazole propionic acid for the entire test solution

which shows that 8.15 per cent of the histidine was converted into this substance.

We wish to call attention here, to the close check between the histidine values obtained by the Van Slyke and the colorimetric methods. The entire significance of this agreement will be discussed in detail at the end of the article. This agreement was not so close in No. 17 where glucose was used in place of glycerol. The products formed by the metabolism of glucose interfere with the color production. This is proved quite clearly by the discrepancy between the color values obtained from 0.20 and 0.10 cc. of the solution. Since 0.10 cc. of the solution had a color value equivalent to 5.3 mm. (CR-MO), 0.20 cc. should have had a color value equivalent to 10.6 mm. The value actually obtained was 8.2 mm. which shows that the color production had been seriously interfered with. A smaller interference would be expected from 0.10 cc. because less of the objectionable substances were introduced. For that reason the calculations were based upon the color value for 0.10 cc.

Histamine Fraction.—This was obtained by extracting the combined amyl alcohol extracts with 1.0 N H_2SO_4 as has already been described under Sections III and VII of the previous paper. The final volume of the neutral solution was always 100 cc. The colorimetric determinations were made on this solution without further dilution. That portion of the histamine fraction which was not used for the colorimetric determinations—99 cc. or more—was treated with 5 cc. of M Na_2CO_3 solution and evaporated to dryness on the water bath. An excess of 37 per cent HCl was then added and the liquid reevaporated to remove the water and acid entirely. The residue was transferred to a graduated precision cylinder with enough water to give a final volume of 25 cc. This concentrated solution, which was now free from ammonia and volatile amines, was used for the following determinations.

1. An Amino Nitrogen Determination.—This was carried out in only a few cases because the presence of about 10 per cent of histamine was regularly indicated by the Van Slyke method when the colorimetric determination showed this substance to be entirely absent. We have found more recently (see Sections II and III of Paper III) that the Van Slyke method can be used for the estimation of histamine when that substance is produced in large

amounts. The entire significance of these facts will be discussed in detail at the end of this section.

2. A Color Value Determination.—In case this determination agreed with the previous determination it served as a check, or if it had been lower than the previous determination it would have *indicated* the presence of methyl imidazole. The redetermined color value always agreed perfectly with that previously determined, which proved that methyl imidazole was regularly absent.

3. A biological verification of the presence of histamine.

4. A chemical verification of the presence of histamine.

The results are given in Table III.

TABLE III.

Histamine Fraction.

Flask No.	Amount of solution used.	Color value of (CR-MO) indicator.	Histamine. In test solution.	Histamine. Histidine converted.	Histidine converted into methyl imidazole by Methods B and C.
	cc.	*mm.*	*gm.*	*per cent*	*per cent*
2	1.0	3.0 Brown.	0.0009	0.45	None.
4	1.0	4.0 Too yellow.	0.00125	0.6	"
5	1.0	4.0 " "	0.00125	0.6	"
6	1.0	3.8 " "	0.0012	0.6	"
7	0.05	11.2	0.075	46.4	"
	0.10	22.2			
8	0.10	6.6	0.0221	13.7	"
	0.20	13.1			
10	1.0	3.8 Too yellow.	0.0012	0.6	"
12	1.0	4.0 " "	0.00125	0.6	"
13	0.05	12.4	0.0825	51.0	"
	0.10	24.7			
14	0.20	6.4	0.01075	6.7	"
	0.40	12.8			
15	0.05	12.1	0.0807	50.0	"
	0.10	24.2			
16	0.10	7.5	0.025	15.5	"
	0.20	15.0			
17	0.10	12.6	0.0425	26.3	"
	0.20	25.3			

Discussion.—The color value of the histamine fraction never was nil. Values lower than 4.0 mm. (CR-MO) for 1.0 cc. of the solution were due to *histidine* not *histamine* (see p. 525). The constancy of this value in those cases where histamine was absent is striking. The color development for histamine is characteristic of that substance and is hard to confuse with that of either histidine or methyl imidazole. In those seven cases where histamine is recorded as present, the color was identical with that obtained from pure histamine. Further proof of the correctness of this method as a means of determining histamine is given below.

Biological Verification of the Presence of Histamine.

A portion of the solution, which had been shown to contain 1 mg. of histamine dichloride by the colorimetric method, was injected into the femoral vein of a dog that had been previously anesthetized and prepared so that a blood pressure and respiratory tracing could be obtained at the same time. Figs. 1 and 2 are representative tracings. Similar tracings were obtained from Samples 7, 8, 13, 14, and 17. The entire Solution 5, which can be taken as a representative of those solutions in which a color value of 4.0 mm. (CR-MO) per 1 cc. was obtained, was injected. There was no apparent change either in blood pressure or respiration. The curve for No. 15 shows an effect that is usually not obtained. The injection was started and interrupted after a fraction of a drop of the solution had entered the circulation. Although this small quantity of substance produced a marked bronchial spasm, it raised the blood pressure very slightly. The subsequent injection of the remainder of the mg. of substance produced the customary drop in blood pressure, together with an intense bronchial spasm.

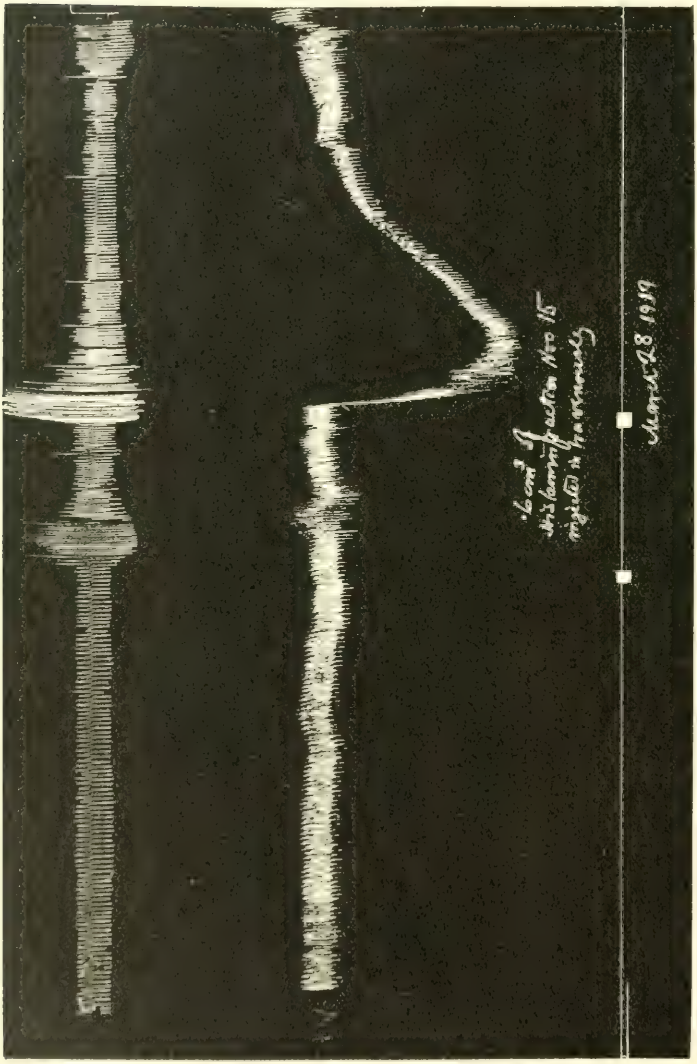


Fig. 1. Respiratory and blood pressure tracings obtained by injecting 0.60 cc. of Histamine fraction No. 15—1.0 mg. of histamine dichloride by colorimetric determination—into the femoral vein of a dog.

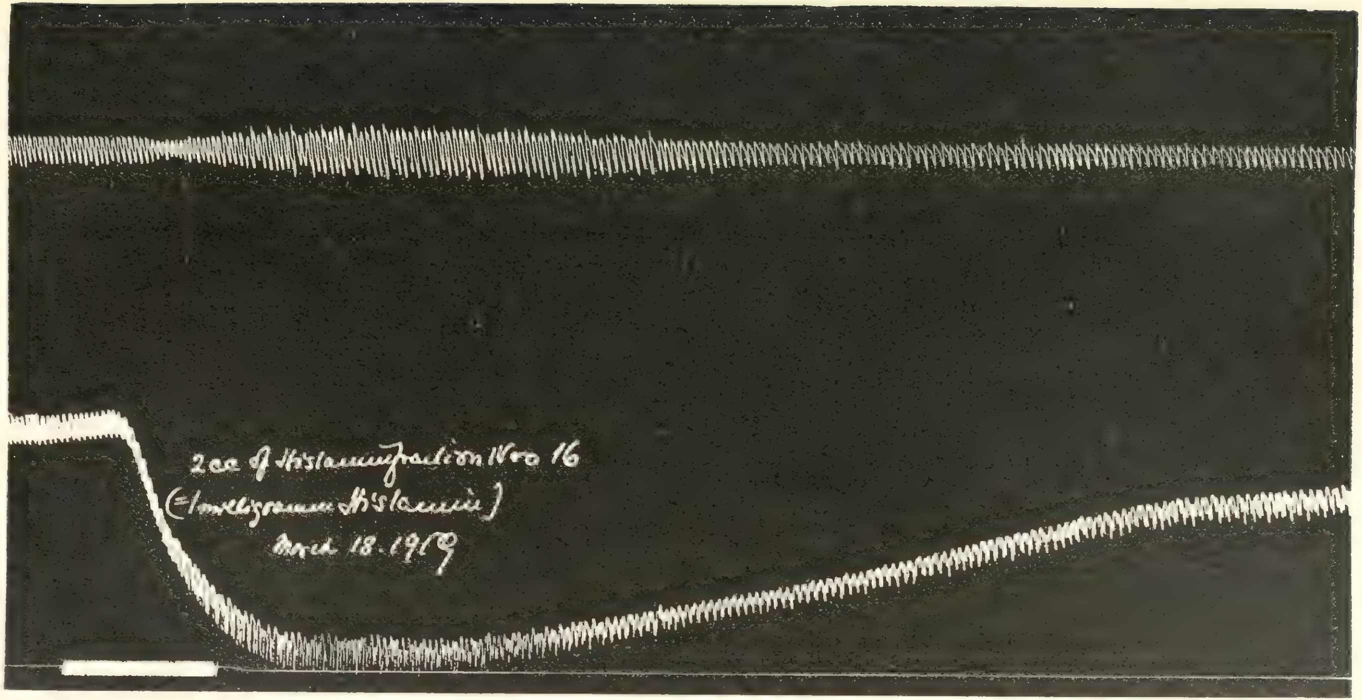


Fig. 2. Respiratory and blood pressure tracings obtained by injecting 2 cc. of Histamine fraction No. 16—1.0 mg. of histamine dichloride by colorimetric determination—into the femoral vein of a dog.

Chemical Verification of the Presence of Histamine.

The remainder of Solutions 7, 8, 13, 14, 15, and 16, whose combined histamine content was 0.10 gm. by colorimetric determination, was united, made strongly alkaline with sodium hydroxide, and extracted with amyl alcohol in the customary manner. The amyl alcohol was then extracted exhaustively with 6 N HCl. The acid extracts were combined and evaporated on the water bath. An entirely solid, pale brown residue was left which was largely sodium chloride. A melting point determination was carried out on a portion of it. A slight softening occurred at 195°. No further change was noticeable until the temperature had risen to 242° (corrected) when an abrupt softening occurred. The solid did not, of course, melt completely because of the presence of sodium chloride. The recorded melting point of histamine dichloride is 244–246° (corrected.)

The solid gave 0.230 gm. of dipicrate in the form of rhombic leaflets, M.P. 238–240°, from 100 cc. of water. This is the recorded melting point of histamine dipicrate. Some of this solid was mixed with an equal portion of pure synthetic histamine dipicrate. The melting point of the mixture was 238–240°. This proves conclusively that the solid was histamine dipicrate.

A further proof of the quantitative exactness of the colorimetric method can be cited. 0.10 gm. of histamine dichloride should give 0.31 gm. of dipicrate. We have found that the solubility of histamine dipicrate in 100 cc. of water is 0.0716 gm. at 8°, 0.077 at 20°, and 1.4 at 95°. Since this picrate was filtered from a solution whose temperature was about 10°, 0.07 gm. of dipicrate was left in the 100 cc. of solvent. Since 0.23 gm. of dipicrate was actually isolated, 0.30 gm. must have been formed in all which agrees very well with the 0.31 gm. demanded by the theory.

Determination of Ammonia and Total Amino Nitrogen in the Test Solution.

Ammonia was determined by the method given in Section VII of Paper III.

The amino nitrogen determinations referred to here were carried out only on those solutions whose total color value was almost nil. In these cases 10 cc. of the test solution were evapo-

rated on the water bath with 5 cc. of M Na_2CO_3 solution until the liquid was free from ammonia and volatile amines. (These solutions always had a very disagreeable amine-like odor when alkaline which disappeared after the liquid had been heated for some time.) An excess of hydrochloric acid was then added and the liquid reevaporated until all the water and acid had disappeared. The solid was transferred to a precision cylinder with enough water to give a final volume of 10 cc. 5 cc. of this solution were then used for the amino nitrogen determinations. The results are given in Tables IV and V.

TABLE IV.

Ammonia and Total Amino Nitrogen.

Flask No.	0.1 N HCl neutralized by NH_3 from 5 cc. of test solution.	Total nitrogen as NH_3.*	N_2 from 5 cc. of test solution (Van Slyke method).			Total N as amino N.
			Amount	Temperature	Pressure	
	cc.	*per cent*	*cc.*	°C.	*mm.*	*per cent*
1	3.51	66.8	1.75	23	753	13.2
2	1.52	28.9				
3	10.38	82.5	1.97	19	744	6.28 (15.0 per cent of histidine N).
4	6.75	53.8				
5	0.10	1.9				
6	0.10	1.9				
7	3.50	27.9				
8	4.46	35.5				
9	3.95	75.0	1.25	20	748	9.5 (also histidine N).
10	0.50	9.5				
11	8.30	66.2	1.66	20	756	5.35 (12.8 per cent of histidine N).
12	5.80	46.2				
13	0.10	1.9				
14	0.90	17.1				
15	5.68	45.3				
16	6.48	51.7				
17	8.15	65.0				

* N_2 in KNO_3 was ignored.

TABLE

Summary of

Flask No.	Composition of completed medium: incubated 14 days at 37°.	Total color value of test solution as histidine Cl_2.* (0.20 gm = 100 %.)	Color value of histidine fraction as histidine Cl_2.* (0.20 gm. = 100 %.)	Unchanged histidine (Van Slyke method) with 5 cc. of test solution.	Histidine converted into imidazole propionic acid (by difference).
					per cent
1. Aerobic.	10 cc. 2 % histidine Cl_2. 100 cc. Salt Medium 1. 90 cc. distilled water.	1.0 cc. = 0.02 mm. Color brown; match poor. **2%**			0
2. Anaerobic.	Same as No. 1.	0.1 cc. = 6.6 mm. 0.2 " = 13.2 " Match perfect. **66.0 %**	0.1 cc. = 6.4 mm. 0.2 " = 12.7 " Match perfect. **64.0 %**	1.13 cc. N_2 at 20° and 748 mm. 0.1290 gm. histidine Cl_2. **64.5 %**	0
3. Aerobic.	10 cc. 2 % histidine Cl_2. 100 cc. Salt Medium 1. 10 cc. 2 % NH_4Cl. 80 cc. distilled water.	1.0 cc. = 3.5 mm. Color brown; match poor. **3.5 %**			0
4. Anaerobic.	Same as No. 3.	0.1 cc. = 10.1 mm. 0.2 " = 20.3 " Match perfect. **98.0 %**	0.10 cc. = 10.1 mm. 0.20 " = 19.8 " Match perfect. **98.0 %**	1.8 cc. N_2 at 22° and 741 mm. 0.2008 gm. histidine Cl_2. **100.4 %**	0
5. Aerobic.	10 cc. 2 % histidine Cl_2. 4 cc. glycerol. 100 cc. Salt Medium 1. 86 cc. distilled water.	0.1 cc. = 9.0 mm. 0.2 " = 18.0 " Match perfect. **90.0 %**	0.10 cc. = 7.9 mm. 0.20 " = 15.8 " Match perfect. **79.0 %**	1.42 cc. N_2 at 20° and 752 mm. 0.1629 gm. histidine Cl_2. **81.5 %**	0

V.

Results. Part I.

Color value of histamine fraction.*	Histidine converted into histamine (colorimetric determination).	Histidine converted into methyl imidazole (Methods B and C).	Amount of 0.1 N HCl neutralized by ammonia from entire test solution.	Total N as NH_3 (N_2 in KNO_3 ignored).	Total N as amino nitrogen with 5 cc. of test solution.	Reaction.	
						Before incubation.	After incubation.
		per cent	*cc.*	*per cent*		*pH*	*pH*
	Histamine absent.	0	17.55	66.8	1.75 cc. N_2 at 23° and 753 mm. 13.2 %.	7.35	8.3
1.0 cc. = 3.0 mm. Color too yellow; match poor.	≈ 0.0009 gm. histamine in test solution. 0.45 % Histamine absent.	0	7.6	28.9		7.35	7.6
	Histamine absent.	0	51.9	82.5	1.97 cc. N_2 at 19° and 744 mm. 6.28 % total N (15.0 % histidine N).	7.35	8.2
1.0 cc. = 4.0 mm. Color too yellow; match poor.	≈ 0.00125 gm. histamine in test solution. 0.60 % Histamine absent.	0	33.75	53.8		7.35	7.5
1.0 cc. = 4.0 mm. Color too yellow; match poor.	≈ 0.00125 gm. histamine in test solution. 0.60 % Histamine absent.	0	0.50	1.9		7.35	7.15

TABLE V

Summary of

Flask No.	Composition of completed medium; incubated 14 days at 37°.	Total color value of test solution as histidine Cl_2.* (0.20 gm. = 100 %.)	Color value of histidine fraction as histidine Cl_2.* (0.20 gm. = 100 %.)	Unchanged histidine (Van Slyke method) with 5 cc. of test solution.	Histidine converted into imidazole propionic acid (by difference).
					per cent
6. Anaerobic.	Same as No. 5.	0.10 cc. = 10.4 mm. 0.20 " = 20.8 " Match perfect. **104.0 %**	0.10 cc. = 7.4 mm. 0.20 " = 14.8 " Too pink. **74.0 %**	1.05 cc. N_2 at 25° and 745 mm. 0.1162 gm. histidine Cl_2. **58.1 %**	8.15
7. Aerobic.	10 cc. 2 % histidine Cl_2. 10 cc. 2 % NH_4Cl. 4 cc. glycerol. 100 cc. Salt Medium 1. 76 cc. distilled water.	0.10 cc. = 9.4 mm. 0.20 " = 18.8 " Match perfect. **94.0 %**	0.10 cc. = 4.6 mm. 0.20 " = 9.2 " Match perfect. **46.0 %**	0.80 cc. N_2 at 20° and 754 mm. 0.092 gm. histidine Cl_2. **46.0 %**	0
8. Anaerobic.	Same as No. 7.	0.10 cc. = 10.0 mm. 0.20 " = 20.0 " Match perfect. **100.0 %**	0.10 cc. = 6.4 mm. 0.20 " = 12.7 " Match perfect. **64.0 %**	1.18 cc. N_2 at 24° and 742 mm. 0.131 gm. histidine Cl_2. **65.5 %**	0
9. Aerobic.	10 cc. 2 % histidine Cl_2. 5 cc. 2 % KNO_3. 100 cc. Salt Medium 1. 85 cc. distilled water.	1.0 cc. = 3.7 mm. Color brown; match poor. **3.7 %**			0
10. Anaerobic.	Same as No. 9.	0.10 cc. = 9.1 mm. 0.20 " = 18.2 " Match perfect. **91.0 %**	0.10 cc. = 8.9 mm. 0.20 " = 17.8 " Match perfect. **89.0 %**	1.60 cc. N_2 at 25° and 753 mm. 0.1792 gm. histidine Cl_2. **89.6 %**	0

–*Continued.*

[R]*esults. Part I.*

Color value of histamine fraction.*	Histidine converted into histamine (colorimetric determination).	Histidine converted into methyl imidazole (Methods B and C).	0.1 N HCl neutralized by ammonia from entire test solution.	Amount of total N as NH_3 (N_2 in KNO_3 ignored)	Total N as amino nitrogen with 5 cc. of test solution.	Reaction.	
						Before incubation.	After incubation.
		per cent	*cc.*	*per cent*		*pH*	*pH*
1.0 cc. = 3.8 mm. Color too yellow; match poor.	≈ 0.0012 gm. histamine in test solution. 0.6 % Histamine absent.	0	0.50	1.9		7.35	7.45
0.05 cc. = 11.2 mm. 0.10 " = 22.2 " Color identical with that obtained with histamine.	≈ 0.075 gm. histamine in test solution. **46.4 %** of histamine present.	0	17.5	27.9		7.35	>6.6
[0].10 cc. = 6.6 mm. [0].20 " = 13.1 " Color identical with that obtained with histamine.	≈ 0.0221 gm. histamine in test solution. **13.7 %** of histamine present.	0	22.3	35.5		7.35	>6.6
	Histamine absent.	0	19.75	75.0	1.25 cc. N_2 at 20° and 748 mm. 9.5 % (ignoring KNO_3).	7.35	8.4
[1].0 cc. = 3.8 mm. [C]olor too yellow; match poor.	≈ 0.0012 gm. histamine in test solution. 0.6 % Histamine absent.	0	2.5	9.5		7.35	8.0

TABLE V

Summary of

Flask No.	Composition of completed medium; incubated 14 days at 37°.	Total color value of test solution as histidine Cl_2.* (0.20 gm. = 100 %.)	Color value of histidine fraction as histidine Cl_2.* (0.20 gm. = 100 %.)	Unchanged histidine (Van Slyke method) with 5 cc. of test solution.	Histidine converted into imidazole propionic acid (by difference).
					per cent
11. Aerobic.	10 cc. 2 % histidine Cl_2. 5 cc. 2 % KNO_3. 10 cc. 2 % NH_4Cl. 100 cc. Salt Medium 1. 75 cc. distilled water.	1.0 cc. = 2.7 mm. Color brown; match poor. **2.7 %**			0
12. Anaerobic.	Same as No. 11.	0.10 cc. = 10.0 mm. 0.20 " = 20.0 " Match perfect. **100.0 %**	0.10 cc. = 9.8 mm. 0.20 " = 19.7 " Match perfect. **98.0 %**	1.75 cc. N_2 at 20° and 750 mm. 0.20 gm. histidine Cl_2. **100.0 %**	0
13. Aerobic.	10 cc. 2 % histidine Cl_2. 5 cc. 2 % KNO_3. 1 cc. glycerol. 100 cc. Salt Medium 1. 84 cc. distilled water.	0.10 cc. = 10.0 mm. 0.20 " = 20.0 " Match perfect. **100.0 %**	0.10 cc. = 4.0 mm. 0.20 " = 8.0 " Match perfect. **40.0 %**	0.70 cc. N_2 at 23° and 753 mm. 0.0791 gm. histidine Cl_2. **39.5 %**	0
14. Anaerobic.	Same as No. 13.	0.10 cc. = 9.7 mm. 0.20 " = 19.4 " Match perfect. **97.0 %**	0.10 cc. = 9.0 mm. 0.20 " = 18.1 " Match perfect. **90.0 %**	1.6 cc. N_2 at 23° and 753 mm. 0.1807 gm. histidine Cl_2. **90.4 %**	0

—*Continued.*

Results. Part 1.

Color value of histamine fraction.*	Histidine converted into histamine (colorimetric determination).	Histidine converted into methyl imidazole (Methods B and C).	0.1 N HCl neutralized by ammonia from entire test solution.	Amount of total N as NH_3 (N_2 in KNO ignored)	Total N as amino nitrogen with 5 cc. of test solution.	Reaction.	
						Before incubation.	After incubation.
		per cent	*cc.*	*per cent*		*pH*	*pH*
	Histamine absent.	0	41.5	66.2	1.66 cc. N_2 at 20° and 756 mm. 5.35 % total N (12.8 % histidine N).	7.35	8.3
1.0 cc. = 4.0 mm. Color too yellow; match poor.	≈ 0.00125 gm. histamine in test solution. 0.60 % Histamine absent.	0	29.0	46.2		7.35	7.9
0.05 cc. = 12.4 mm. 0.10 " = 24.7 " Color identical with that obtained with histamine.	≈ 0.0825 gm. histamine in test solution. **51.0 %** of histamine present.	0	0.50	1.9		7.35	>6.6
0.20 cc. = 6.4 mm. 0.40 " = 12.8 " Color identical with that obtained with histamine.	≈ 0.01075 gm. histamine in test solution. **6.7 %** of histamine present.	0	4.5	17.1		7.35	>6.6

TABLE V

Summary of

Flask No.	Composition of completed medium; incubated 14 days at 37°.	Total color value of test solution as histidine Cl_2.* (0.20 gm. = 100 %.)	Color value of histidine fraction as histidine Cl_2.* (0.20 gm. = 100 %.)	Unchanged histidine (Van Slyke method) with 5 cc. of test solution.	Histidine converted into imidazole propionic acid (by difference).
					per cent
15. Aerobic.	10 cc. 2 % histidine Cl_2. 5 cc. 2 % KNO_3. 10 cc. 2 % NH_4Cl. 4 cc. glycerol. 100 cc. Salt Medium 1. 71 cc. distilled water.	0.10 cc. = 10.0 mm. 0.20 " = 20.0 " Match perfect. **100.0 %**	0.10 cc. = 4.5 mm. 0.20 " = 9.0 " Match perfect. **45.0 %**	0.85 cc. N_2 at 23° and 754 mm. 0.0961 gm. histidine Cl_2. **48.0 %**	0
16. Anaerobic.	Same as No. 15.	0.10 cc. = 10.0 mm. 0.20 " = 20.0 " Match perfect. **100.0 %**	0.10 cc. = 7.5 mm. 0.20 " = 15.0 " Match perfect. **75.0 %**	1.35 cc. N_2 at 20° and 753 mm. 0.1549 gm. histidine Cl_2. **77.4 %**	0
17. Aerobic.	10 cc. 2 % histidine Cl_2. 5 cc. 2 % KNO_3. 10 cc. 2 % NH_4Cl. 5 gm. glucose. 100 cc. Salt Medium 1. 70 cc. distilled water.	0.10 cc. = 9.3 mm. 0.20 " = 16.0 " Match good. **93.0 %**	0.10 cc. = 5.3 mm. 0.20 " = 8.2 " Match good. **53%**	1.06 cc. N_2 at 24° and 737 mm. 0.1168 gm. histidine Cl_2. **58.4 %**	0

* Colors matched against the (CR–MO) standard.

-Concluded.

Results. Part I.

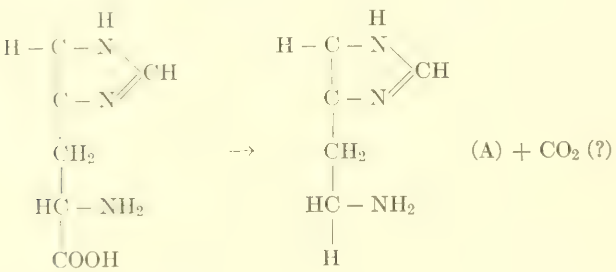
Color value of histamine fraction.*	Histidine converted into histamine (colorimetric determination).	Histidine converted into methyl imidazole (Methods B and C).	0.1 N HCl neutralized by ammonia from entire test solution.	Amount of total N as NH_3 (N_2 in KNO_3 ignored).	Total N as amino nitrogen with 5 cc. of test solution.	Reaction.	
						Before incubation.	After incubation.
		per cent	*cc.*	*per cent*		*pH*	*pH*
= 0.05 cc. = 12.1 mm. 0.10 " = 24.2 " Color identical with that obtained with histamine.	0.0807 gm. histamine in test solution. **50.0 %** of histamine present.	0	28.4	45.3		7.35	>6.6
= 0.10 cc. = 7.5 mm. 0.20 " = 15.0 " Color identical with that obtained with histamine.	0.025 gm. histamine in test solution. **15.5 %** of histamine present.	0	32.4	51.7		7.35	>6.6
= 0.10 cc. = 12.0 mm. 0.20 " = 25.3 " Color identical with that obtained with histamine.	0.0425 gm. histamine in test solution. **26.3 %** of histamine present.	0	40.75	65.0		7.35	>6.6

Theoretical Considerations. Discussion of Results.

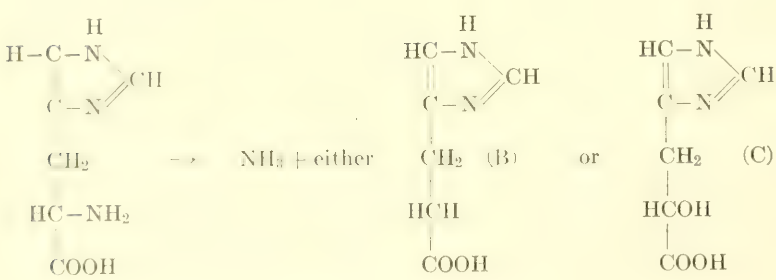
A consideration of the experiments given in Tables IV and V together with those of the other authors referred to in the introduction has led us to the following tentative theoretical deductions.

It should be possible for histidine to undergo four kinds of *primary decomposition.*

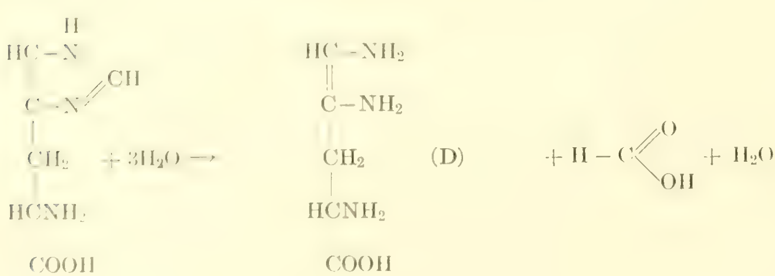
Type 1. Decarboxylation.



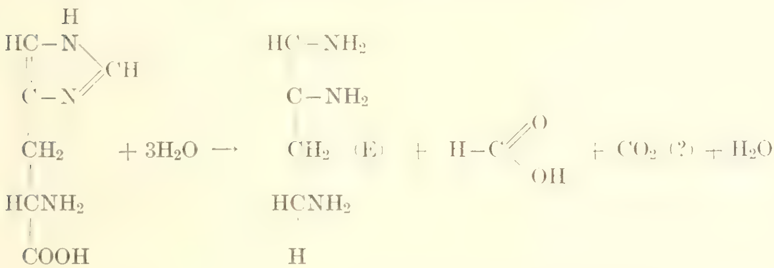
Type 2. Deamination.



Type 3. Nuclear disruption without decarboxylation.

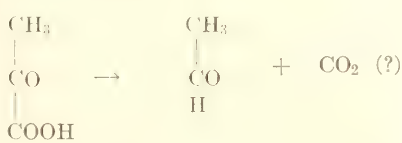


Type 4. Nuclear disruption with decarboxylation.



We have now to consider under what conditions each type of decomposition is to be expected.

Type 1.—We can see only two reasons why a microorganism would decarboxylate histidine: (1) To obtain the particular kind of carbon that is present in the carboxyl group ($^{+}_{+}C^{+}_{+}$ but more probably $^{+}_{-}C^{+}_{+}$); and (2) to produce the base histamine with which excess of acidity could be neutralized more efficiently than with ammonia. Decarboxylation would lead to a lowering of the hydrogen ion concentration of the solution irrespective of whether a base was formed or not because the organic acids are usually more highly dissociated than carbonic acid. Thus the decarboxylation of pyruvic acid, for example, would lead to a reduction of the hydrogen ion concentration of the solution even though a base was not formed.



We have placed a (?) behind the CO_2 in each case because we are not certain that the carboxyl group appears immediately as CO_2, or, indeed, whether it appears in this form at all. It is possible that formic acid is first formed which later gives CO_2 by oxidation.

This type of decomposition would then be expected when another source of carbon was available and when the hydrogen ion concentration of the medium threatened to become too high for the proper development of the organism.

Type 2.—Histidine might be deaminized for three reasons.

1. The nitrogen might be removed because it was necessary for the proper development of the organism. This would be expected to occur when other available sources of nitrogen, such as ammonium salts or nitrates, were absent. In the presence of an easily available source of carbon but an inadequate source of nitrogen, the decomposition of the histidine molecule might easily cease after the nitrogen had been removed. Under these conditions imidazole lactic or propionic acids might be present unchanged at the end of the experiment.

2. The nitrogen might be removed not because it was needed, but because its removal was a necessary precursor or adjunct to the utilization of the aliphatic carbon chain. Such a decomposition might occur when other available sources of carbon were absent irrespective of the presence or absence of a nitrogen source.

3. Deamination might be resorted to as a means of lowering the hydrogen ion concentration of the medium. The equation given under Type 2 shows that the mere removal of the ammonia from the amino-acid molecule can have but little effect upon the hydrogen ion concentration of the medium because the acid radical is still present and it will neutralize the ammonia at least as completely as it did the amino group. A subsequent oxidation of the aliphatic carbon chain would destroy this acid group, however, and then the ammonia would be of service as a neutralizer of acidity. This process would be a more roundabout method for reducing the pH of a medium than direct decarboxylation. There is little doubt that this process is made use of by the body cells, and some bacteria probably proceed in the same manner.

Types 3 and 4.—A nuclear disruption, either with or without decarboxylation, is the most certain way to expose for future use all the nitrogen and carbon of the molecule. This is also a decomposition that converts a feebly basic substance into one that is strongly basic. This type of disruption might be expected if either the solution contained no other source of carbon or nitrogen than histidine or the initial pH of the medium was so markedly unfavorable to the organism that it was necessary to liberate a large amount of a base in the shortest possible time. Since a microorganism requires far more carbon than nitrogen for its growth and metabolism, a nuclear rupture would be expected to

occur even in the presence of a large excess of ammonium salts or nitrates, when the medium contained no more available source of carbon than histidine.

It is also to be expected that a substance of Type E, containing no carboxyl group, will pass into the amyl alcohol layer during the extraction process, while one of Type D, containing a carboxyl group, will remain in the alkaline aqueous layer along with the histidine.

We will now pass to a discussion of the individual cases and attempt to explain the results obtained on the basis of these theoretical deductions.

Flask 1.—In this case histidine was the only source of carbon and nitrogen. An examination of Table V shows that (1) the imidazole ring was destroyed to the extent of 98 per cent; (2) about 67 per cent of the total, not amino, nitrogen of the molecule was converted into ammonia; (3) about 13 per cent of the total nitrogen was still in the amino condition; and (4) the pH changed from 7.35 to 8.3. These results can be explained as follows.

The only source of carbon and nitrogen was histidine. A nuclear rupture would expose these elements so that they could serve as foods. Since very little of the nitrogen was required as compared to the carbon, most of it was eliminated as ammonia which lowered the hydrogen ion concentration of the solution.

Flask 2.—The composition of this medium was identical with that of No. 1 except that the oxygen concentration was almost nil. The nucleus was ruptured only to the extent of 34 per cent. The histidine that did not undergo a nuclear cleavage was untouched. About 29 per cent of the total nitrogen appeared as ammonia. The reaction of the medium changed from pH 7.35 to 7.6. The close check between the amino nitrogen and colorimetric values for histidine shows that molecules of Type D were absent. An amino nitrogen determination on the histamine fraction, which was carried out after the solution had been freed from ammonia and volatile amines, showed that 4 per cent of the total nitrogen was present in this form. This indicates that compounds of Type E were present. A nuclear rupture with decarboxylation occurred in this case which proves that *the colon bacillus has the power of decarboxylating acids, which suggests that under proper conditions it may be able to make amines from amino-acids.* All the

nitrogen introduced was accounted for. Thus 66 per cent of the histidine was recovered; 29 per cent of the nitrogen appeared as ammonia; 4 per cent was non-volatile amino nitrogen in the histamine fraction, which makes a total of 99 per cent. In this case, then, volatile amines were absent.

The explanations offered for No. 1 apply also in this case. We have found that the anaerobic differed from the aerobic experiments in quantity, not in quality. The rate of metabolism is much reduced by the oxygen deficit but the quality of the products is not affected.

Flask 3.—The results obtained in this case were almost identical with those obtained for No. 1, with the apparent exception of the ammonia value. Such an amount of ammonium chloride was originally introduced, however, that the ammonia from the entire test solution should have neutralized 36.40 cc. of 0.1 N acid. When this is subtracted from the 51.9 cc. required by experiment, 15.5 cc. of 0.1 N acid are found to have been used to neutralize the ammonia liberated from the histidine which compares very well with the 17.55 cc. required for No. 1.

The analogous results obtained in Nos. 1 and 3 can be explained by the fact that although the organism was supplied plentifully with a nitrogen source, histidine was still the only source of carbon. A nuclear rupture followed by deamination was the surest way to make this carbon available.

Flask 4.—Table V shows that 98 per cent of the histidine was recovered unchanged. The almost complete absence of metabolic activity led us to believe that the organisms had died. This proved not to be the case. This is merely another example of the necessity of oxygen for the metabolism of the colon bacillus.

Flask 5.—In this case histidine was the only source of nitrogen but glycerol was added as a source of carbon. About 80 per cent of the histidine was recovered. Here again there was a complete absence of molecules of Type D. Only 2 per cent of the histidine nitrogen was recovered as ammonia. For the first time we were confronted with a peculiar discrepancy. The total color value determination indicated that about 90 per cent of the imidazole ring was still intact. Only 80 per cent of it was proved to be histidine and the remaining 10 per cent disappeared in the extraction process. We are tempted to conclude that some heterocyclic

compound of undetermined constitution was present that was completely destroyed by the strong alkali used in the extraction process. This conclusion is rendered still more plausible by the subsequent experiments.

Imidazole lactic, propionic, or acetic acids were absent which, of course, means that they were either not formed under these conditions or subsequently oxidized.

In the presence of an available carbon source the histidine molecule was called upon merely as a source of nitrogen. The nitrogen was obtained partially by deamination and perhaps to a small extent by a partial rupture of the nucleus. The amount of nitrogen liberated exceeded the metabolic requirements of the organism by only 2 per cent.

Flask 6.—The composition of this medium differed from that of No. 5 only in the atmospheric oxygen content, which in this case was almost nil. Of the entire series, this is the one case in which the formation of imidazole propionic acid was indicated. Only 2 per cent of the histidine nitrogen was recovered as ammonia. Here again we were confronted with the same type of color value discrepancy that was noted with No. 5. The total color value determination indicated the presence of 100 per cent of the original imidazole ring. Only 74 per cent of this color value was accounted for as histidine and imidazole propionic acid; about 25 per cent was lost in the extraction process. This again indicates the formation of an alkali-labile heterocyclic compound. A discrepancy of this kind was obtained in only one other case (No. 8) where it amounted to 20 per cent. It seems that the labile compound is formed more easily or destroyed less easily under anaerobic conditions.

The absence of oxygen would hinder oxidation reaction and favor reductions. The formation of imidazole propionic acid from histidine is a reduction regardless of what assumptions are made as to the process.

$$\begin{array}{c} \mathrm{R} \\ | \\ \mathrm{H-C} \\ | \\ \mathrm{COOH} \end{array} + -\mathrm{NH_2} + 2(\epsilon) + 2(\mathrm{H}) \longrightarrow \begin{array}{c} \mathrm{R} \\ | \\ \mathrm{H-C-} \\ | \\ \mathrm{COOH} \end{array} + \mathrm{H} + \mathrm{NH_3}$$

There is no doubt that, in this case, deamination was resorted to by the colon bacillus as a means of obtaining nitrogen. To judge from the total color value determination, a nuclear disruption did not occur. It appears that this organism is readily capable of rupturing the imidazole ring only in the presence of oxygen.

Flask 7.—This medium contained both an easily available source of carbon and nitrogen. Table V shows that: (1) this is the first member of the series that was distinctly acid after incubation; (2) 46.4 per cent of the histidine was converted into histamine; (3) unchanged histidine was the only other product; therefore (4) a marked nuclear rupture did not occur; and (5) about 50 per cent of the ammonia that was originally introduced was consumed by the bacteria.

The histidine in this medium was unnecessary as a food constituent because the organisms grew perfectly with just the glycerol and ammonium salt to serve as carbon and nitrogen sources. Under these conditions neither a nuclear disruption nor deamination could be expected unless a nuclear rupture was resorted to as a means of neutralizing excess acidity. Decarboxylation occurred to a very marked extent—46.4 per cent—which proves that the colon bacillus has this power. We concluded (see Theoretical Considerations) that the organism would decarboxylate histidine either to get the carboxyl carbon or to manufacture the strong base histamine. This experiment leaves us largely in the dark as to the purpose of the decarboxylation. The fact that histamine was never found, in our experiments, unless the medium had become distinctly acid, suggests strongly that the base was formed merely to lower the hydrogen ion concentration of the solution. In Part III of this paper, proof that this is actually the case is presented.

Flask 8.—This was the anaerobic equivalent of No. 7. Table V shows that (1) the medium was distinctly acid after incubation; (2) 13.7 per cent of the histidine was converted into histamine as compared to 46.4 per cent in No. 7; (3) unchanged histidine was the only other recognizable product so that here again there is the indication that an alkali-labile heterocyclic compound was formed to the extent of 20 per cent; (4) there was no evidence of a nuclear disruption; (5) about 40 per cent of the ammonia that was originally introduced was removed from the solution by the bacteria.

The discussion given for No. 7 applies also to this case. The oxygen deficit decreased the rate of metabolism.

Flasks 9 to 16 inclusive.—The remaining experiments were duplicates of those already described except that potassium nitrate was present in all the media. We introduced the nitrate to see whether it would have any effect upon the production of histamine. Potassium nitrate might be expected to function in one or more of the following ways: (1) as a source of potassium or nitrogen; (2) as a source of alkali and ammonia, both of which might be used for the purpose of neutralizing excess of acidity;

$$KNO_3 + 8(H) \rightarrow KOH + NH_3 + 2H_2O$$

(3) as a source of oxygen;

$$KNO_3 + 2(H) + H_2O \rightarrow KOH + NH_3 + 3(O)$$

In this case the ammonia need not, of course, be liberated as such, but might be used for synthetic purposes.

The results obtained in Flasks 13 and 14 prove that KNO_3 is a very efficient source of nitrogen for the colon bacillus. These results were fairly close duplicates of those obtained in Flasks 7 and 8 in which ammonium chloride was present instead of potassium nitrate. A slightly larger percentage of histamine was always formed in the presence of the nitrate than in the presence of the ammonium salt. Thus Flasks 15 and 16 contained more histamine than Flasks 7 and 8 respectively.

In Flask 14, some of the KNO_3 must have been converted into ammonia. In this case 97 per cent of the total nitrogen was present either as histidine or histamine. If all the remaining 3 per cent of nitrogen had been converted into ammonia, the entire test solution would have contained enough of it to neutralize only 0.80 cc. of the 0.10 N acid. Since 4.5 cc. of the acid were actually neutralized by ammonia arising from this solution, we must conclude that enough NH_3 to neutralize 3.7 cc. of the acid must have been formed from the KNO_3 which shows that at least 37 per cent of the nitrate was converted into ammonia.

For the colon bacillus, potassium nitrate seems to serve chiefly as a source of nitrogen. With this in mind the results obtained in Experiments 9, 10, 11, and 12 can be readily understood since they agree perfectly with the theoretical stipulations.

Flask 17.—The nutritive conditions were identical, in this case, to those of No. 15 except that glucose was used as a carbon source in place of glycerol. The results obtained may be summarized as follows: (1) the medium was distinctly acid after incubation; (2) 26.3 per cent of the histidine was converted into histamine as compared to 46.4 per cent in No. 7; (3) about 58 per cent of the histidine was unchanged; (4) a nuclear disruption had occurred to the extent of about 15 per cent; and (5) ammonia was generated by the bacteria either from the histidine or from the potassium nitrate. In either case, this ammonia would help to reduce the hydrogen ion concentration of the medium.

Since the nutritive conditions of this experiment were identical with those of No. 15, except for the carbon source, the discovery that 26 per cent of the histidine was converted into histamine would be no surprise if one had not read the literature. The statement is repeatedly made that glucose prevents the decarboxylation of amino-acids by bacteria. Thus Mellanby and Twort,[7] Berthelot and Bertrand,[8] and Jones[11] claim that histamine is not produced in the presence of glucose. The experimental conditions employed by these observers were different from our own and this may account for the difference in results. As will be shown in Parts II and III, such factors as time of incubation and the initial concentration of disodium acid phosphate have a very decided influence upon the formation of histamine from histidine.

There seems to have been a tendency, in the past, to confuse the sparing action of carbohydrates for proteins and the effect of carbohydrates on the decarboxylation of amino-acids. That a body cell or a microorganism will obtain its carbon requirements from carbohydrates by preference to amino-acids or proteins has been proved beyond doubt. Our own experiments show quite conclusively that this principle is correct. Thus in Nos. 1, 3, 9, and 11 where histidine was the only source of carbon, a complete decomposition of the molecule occurred, the histidine being used as a carbon source. When glycerol was added, the histidine remained untouched except as it was called upon to supply nitrogen or to neutralize acidity. The idea that the carbohydrate would also prevent decarboxylation must have arisen because it

[11] Jones, H. M., *J. Infect. Dis.*, 1918, xxii, 125.

was thought that the carboxyl carbon served as a carbon source. If this were actually the case it would be easy to see why the addition of an easily available source of carbon would render decarboxylation unnecessary. The facts are, however, that decarboxylation with amine production occurs only in the presence of an outside source of carbon and that this can be prevented by neutralizing the excess of acidity (see Part III).

These statements apply, of course, only to the colon bacillus. We shall report, at a later date, experiments conducted with other microorganisms and, until these experiments have been completed, a generalization is not admissible.

Part II. The Effect of Duration of Incubation upon Conversion of Histidine into Histamine.

The solutions employed, the mode of procedure, and the methods of analysis were identical with those described in Part I. These experiments were duplicates of No. 15 of Part I except that the time of incubation varied from 2 to 40 days.

The composition of the salt medium did not differ materially from that of Experiment 15. The concentration of the sodium chloride was slightly reduced and the potassium chloride was eliminated. The ammonium chloride and potassium nitrate were incorporated into the salt medium.

Composition of Salt Medium 2.— 4.00 gm. of ammonium chloride.
2.00 " " potassium nitrate.
8.00 " " " dihydrogen phosphate.
16.00 " " sodium chloride.
0.40 " " " sulfate.
8.00 " " " bicarbonate.
0.20 " " calcium chloride.

These amounts were dissolved in water and diluted to exactly 2,000 cc.

Composition of Completed Media.—10 cc. of 2 per cent histidine dichloride.
4 " " glycerol.
100 " " Salt Medium 2.
86 " " distilled water.

These amounts were mixed in a 300 cc. Pyrex Florence flask. The salt medium was added from a 100 cc. normal pipette.

The results are given in Table VI.

TABLE

Summary of

Flask No.	Composition of completed medium.	Time of incubation.	Total color value of test solution as histidine Cl_2.* (0.20 gm. = 100 %.)	Color value of histidine fraction as histidine Cl_2.* (0.20 gm. = 100 %.)	Unchanged histidine (Van Slyke method) with 5 cc. of test solution.	Histidine converted into imidazole propionic acid (by difference)
		days				*per cent*
1	10 cc. 2 % histidine Cl_2. 4 cc. glycerol. 100 cc. Salt Medium 2. 86 cc. distilled water.	2	0.10 cc. = 10.0 mm. 0.20 " = 20.0 " Match perfect. **100.0 %**	0.10 cc. = 9.2 mm. 0.20 " = 18.3 " Match perfect. **92.0 %**	1.70 cc. N_2 at 22° and 746 mm. 0.191 gm. histidine Cl_2. **95.0 %**	0
2	Same as No. 1.	5	0.10 cc. = 10.0 mm. 0.20 " = 20.1 " Match perfect. **100.0 %**	0.10 cc. = 8.7 mm. 0.20 " = 17.5 " Match perfect. **87.0 %**	1.52 cc. N_2 at 22° and 746 mm. 0.1708 gm. histidine Cl_2. **85.5 %**	0
3	" " " 1.	10	0.10 cc. = 10.1 mm. 0.20 " = 20.2 " Match perfect. **101.0 %**	0.10 cc. = 7.4 mm. 0.20 " = 14.8 " Match perfect. **74.0 %**	1.38 cc. N_2 at 24° and 737 mm. 0.152 gm. histidine Cl_2. **76.0 %**	0
4	" " " 1.	14†	0.10 cc. = 10.0 mm. 0.20 " = 20.0 " Match perfect. **100.0 %**	0.10 cc. = 4.5 mm. 0.20 " = 9.0 " Match perfect. **45.0 %**	0.85 cc. N_2 at 23° and 754 mm. 0.0961 gm. histidine Cl_2. **48.0 %**	0
5	" " " 1.	20	0.10 cc. = 11.4 mm. 0.20 " = 23.0 " Color more like that of histamine than histidine. **114.0 %**	0.10 cc. = 1.4 mm. 0.50 " = 7.0 " Match perfect. **14.0 %**	0.33 cc. N_2 at 24° and 750 mm. 0.037 gm. histidine Cl_2. **18.5 %**	0

1.

Results. Part II.

Color value of histamine fraction.*	Histidine converted into histamine (colorimetric determination).	Histidine converted into methyl imidazole (Methods B and C).	Amount of 0.1 N HCl neutralized by ammonia from entire test solution.	Total N as NH_3 (N_2 in KNO_3 ignored).	Per cent of histidine that had been converted into histamine (Van Slyke method). 5 cc. of the test solution gave.	Reaction. Before incubation	Reaction. After incubation.
		per cent	cc.	per cent		pH	pH
1.0 cc. = 4.0 mm. Color greenish yellow; match poor.	Histamine absent.	0	39.75	63.4		7.35	6.8
0.40 cc. = 6.7 mm. Match good.	0.0053 gm. histamine in test solution. **3.3** % of histamine present.	0	39.75	63.4		7.35	Slightly >6.6
0.10 cc. = 11.8 mm. 0.20 " = 23.5 " Match perfect; identical with that obtained with histamine.	0.039375 gm. histamine in test solution. **24.4** % of histamine present.	0	38.25	61.0		7.35	>6.6
0.05 cc. = 12.1 mm. 0.10 " = 24.2 " Match perfect; identical with that obtained with histamine.	0.0807 gm. histamine in test solution. **50.0** % of histamine present.	0	28.4	45.3		7.35	>6.6
0.025 cc. = 9.4 mm. 0.05 " = 18.8 " Match perfect; identical with that obtained with histamine.	0.12625 gm. histamine in test solution. **78.0** % of histamine present.	0	31.5	50.2	1.37 cc. N_2 at 24° and 747 mm. 0.1237 gm. histamine Cl_2. **77.0** %	7.35	>6.6

TABLE VI

Summary of

Flask No.	Composition of completed medium.	Time of incubation.	Total color value of test solution as histidine Cl_2.* (0.20 gm. = 100 %.)	Color value of histidine fraction as histidine Cl_2.* (0.20 gm. = 100 %.)	Unchanged histidine (Van Slyke method) with 5 cc. of test solution.	Histidine converted into imidazole propionic acid (by difference).
		days				*per cent*
6	Same as No. 1.	30	0.10 cc. = 11.3 mm. 0.20 " = 22.5 " Color more like that of histamine than histidine. **113.0 %**	0.50 cc. = 4.0 mm. Match good. **8.0 %**	0.33 cc. N_2 at 24° and 750 mm. 0.037 gm. histidine Cl_2. **18.5 %**	0
7	" " " 1.	40	0.10 cc. = 11.3 mm. 0.20 " = 22.6 " Color more like that of histamine than histidine. **113.0%**	0.50 cc. = 4.0 mm. Match good. **8.0 %**	0.33 cc. N_2 at 21° and 751 mm. 0.0377 gm. histidine Cl_2. **18.8 %**	0

* Colors matched against the (CR–MO) standard.

† Copied from Table V, Flask No. 15.

·Concluded.

·esults. *Part II.*

olor value of histamine fraction.*	Histidine converted into histamine (colorimetric determination).	Histidine converted into methyl imidazole (Methods B and C).	Amount of 0.1 N HCl neutralized by ammonia from entire test solution.	Total N as NH_3 (N_2 in KNO_3 ignored).	Per cent of histidine that had been converted into histamine (Van Slyke method). 5 cc. of the test solution gave.	Reaction. Before incubation.	Reaction. After incubation.
		per cent	*cc.*	*per cent*		*pH*	*pH*
025 cc. = 9.9 mm. 05 " = 19.8 " [atch perfect; identical with that obtained with histamine.	0.1325 gm. histamine in test solution. **82.0** % of histamine present.	0	25.5	40.7	1.40 cc. N_2 at 21° and 752 mm. 0.1291 gm. histamine Cl_2. **80.0** %	7.35	>6.6
025 cc. = 10.1 mm. 05 " = 20.2 " [atch perfect; identical with that obtained with histamine.	0.135 gm. histamine in test solution. **83.5** % of histamine present.	0	20.0	32.0	1.53 cc. N_2 at 24° and 752 mm. 0.139 gm. histamine Cl_2. **86.0** %	7.35	>6.6

Although the table is largely self explanatory, the following facts call for special emphasis.

The organisms multiplied abundantly during the first 2 days. Some acid was produced but not enough to counteract the neutralizing effect of the $KNaHPO_4$. Ammonia was formed either from the nitrate or from the histidine. *Histamine was absent.*

During the next 3 days enough acid had been formed to counteract entirely the neutralizing ability of the phosphate and the generated ammonia. *During this period 3.3 per cent of the histidine was converted into histamine.*

During the next 5 days decarboxylation proceeded rapidly so that a total of 24.4 per cent of the histidine was converted into histamine. The concentration of ammonia began to drop as it was used to supply the nitrogen requirements of the organism.

During the next 10 days there was an enormous increase in the concentration of the histamine so that 78 per cent of the histidine had been decarboxylated during the entire 20 day period.

The next 20 days produced a slight but steady increase in the histamine concentration and a marked decrease in the ammonia concentration. During this period the process of decarboxylation, as a means of neutralizing acidity, seems to have given place to the far more efficient process of nuclear disruption; for although the color value determination indicated the presence of only 8 per cent of histidine, the amino nitrogen determination showed that the equivalent of 18 per cent of histidine was present. This shows that about 3 per cent of the histidine had undergone a nuclear rupture with the formation of a compound of Type D (see p. 564). The absence of appreciable quantities of compounds of Type E was proved by the close check between the colorimetric and the amino nitrogen determinations on the histamine fraction. A rupture of the imidazole ring in the histamine molecule did not occur during these 40 days of incubation.

Finally, we wish again to call attention to the fact that histamine appeared only in the media that had become distinctly acid during the incubation period.

Part III. Histamine May Be Formed by the Colon Bacillus to Lower the Hydrogen Ion Concentration of the Medium.

The solutions employed, the mode of procedure, and the methods of analysis were identical with those described in Part I. The composition of the salt medium was changed so that the glycerol became a part of it, which made it unnecessary to add this substance separately. We repeat the composition of this complete medium so that it may be referred to conveniently in our later reports.

Composition of Nutritive Medium 3.—

4.00	gm.	of ammonium chloride.
2.00	"	" potassium nitrate.
8.00	"	" " dihydrogen phosphate.
16.00	"	" sodium chloride.
0.40	"	" anhydrous sodium sulfate.
8.00	"	" sodium bicarbonate.
0.20	"	" anhydrous calcium chloride.
80.00	cc	" glycerol.

These amounts were dissolved in water and diluted to exactly 2,000 cc.

Composition of the Completed Media.—

No. 1. 10 cc. of 2 per cent histidine dichloride.
100 " " Nutritive Medium 3.
90 " " distilled water.
2.5 gm. of $Na_2HPO_4.12\ H_2O$.

No. 2. 10 cc. of 2 per cent histidine dichloride.
100 " " Nutritive Medium 3.
90 " " distilled water.
3.8 gm. of $Na_2HPO_4.12\ H_2O$.

No. 3. 10 cc. of 2 per cent histidine dichloride.
100 " " Nutritive Medium 3.
90 " " distilled water.
0.50 gm. of sodium formate.

No. 4. 10 cc. of 2 per cent histidine dichloride.
100 " " Nutritive Medium 3.
90 " " distilled water.
1 " " 2.5 per cent filtered litmus solution

DISCUSSION.

The four flasks were incubated at 37° for 14 days. Flask 4 was equipped with a two-hole rubber stopper into which were inserted a dropping funnel and a glass tube that served as an air vent. During the 2 week period, 14 cc. of normal sodium hydroxide and 2 cc. of sterile 2.5 per cent litmus solution were added to the contents of the flask through the funnel. The reaction of the medium was slightly toward the acid side of neutrality all the time and it became distinctly acid a few times. The addition of the litmus solution was necessary because the bacterial action discharged the color of the mixture entirely.

An abundant growth was obtained in all the flasks. The bacteria were alive at the end of the experiment. None of the media was contaminated.

The litmus that remained in Flask 4 at the close of the experiment did not interfere with the colorimetric determinations because practically all of it appeared in the form of a coagulum when the filtered liquid was evaporated with 1 cc. of 95 per cent sulfuric acid.

The results are given in Table VII.

The following facts call for special emphasis and interpretation.

The media employed in these experiments were identical with those of Part II of this paper, except that various sources of alkali were added. In Experiment 4 of Part II, the time of incubation was 14 days which makes it analogous to the experiments now under consideration. In this time interval 50 per cent of the histidine had been converted into histamine.

In Experiment 1 of Part III, where 2.5 gm. of hydrated basic sodium phosphate were added to control the pH of the medium, only 10 per cent of the histidine had been converted into histamine. When, in Experiment 2, the concentration of the phosphate was increased so that a total of 3.8 gm. had been added, only 1 per cent of the histidine was converted into histamine. Some free acid had accumulated in both cases which shows that not enough basic phosphate had been added to neutralize entirely the accumulating acid.

From these experiments one would be tempted to conclude that histamine was produced only after the neutralizing effect of the basic phosphate had been overcome by the accumulating acid.

The initial addition of more phosphate would keep the solution neutral for a longer time; hence would delay the production of histamine. This would seem to indicate strongly that the base histamine was produced from histidine to reduce the pH of the solution. When basic phosphate was added, the production of the amine was rendered unnecessary for a time because it, the phosphate, prevented a rise in the pH of the medium.

It might be claimed, however, that in some way the phosphate inhibited the decarboxylase activity of the microorganism; therefore, Experiment 4 was carried out. In this case sodium hydroxide was added from time to time to keep the solution as nearly neutral as possible. Although a luxuriant growth was obtained, only 1 per cent of the histidine was decarboxylated under these conditions. There can be no doubt, then, that in this case the neutralization of the medium prevented, or, as we might say, rendered unnecessary, the production of histamine.

Experiment 3 was originally carried out to see if the addition of carboxyl carbon in an easily available form would prevent the decarboxylation of histidine. The addition of formic acid in any but very small concentrations was not feasible because it would raise the pH of the medium to such an extent that the organisms would be unable to multiply properly. Sodium formate was, therefore, resorted to. We soon saw, however, that this experiment would not prove the point for the following reasons.

Omelianski[12] has shown that sodium formate is rapidly decomposed by *Bacterium formicum* according to the equation

$$2(HCOONa + H_2O) \rightarrow Na_2CO_3 + H_2O + CO_2 + 2H_2$$

Pakes and Jollyman,[13] and Karczag and Schiff[14] have shown that formic acid is similarly decomposed by the colon bacillus. Since this decomposition is one that proceeds readily and rapidly, sodium formate is really an excellent source of alkali since it yields sodium carbonate when it is acted upon by bacteria. Instead of decarboxylating histidine to obtain the base histamine, sodium formate was decarboxylated which gave rise to the very

[12] Omelianski, W., *Centr. Bacteriol., 2te Abt.*, 1904, xi, 177, 256, 317.

[13] Pakes, W. C. C., and Jollyman, W. H., *J. Chem. Soc.*, 1901, lxxix, 322, 386, 459.

[14] Karczag, L., and Schiff, E., *Biochem. Z.*, 1915, lxx, 329.

Summary of

Flask No.	Composition of completed medium; incubated for 14 days at 37°.	Total color value of test solution as histidine Cl_2.* (0.20 gm. = 100 %.)	Color value of histidine fraction as histidine Cl_2.* (0.20 gm. = 100 %.)	Unchanged histidine (Van Slyke method) with 5 cc. of test solution.	Histidine converted into imidazole propionic acid (by difference).
					per cent
1	10 cc. 2 % histidine Cl_2. 100 cc. Nutritive Medium 3. 90 cc. distilled water. 2.5 gm. Na_2HPO_4. 12 H_2O.	0.10 cc. = 9.3 mm. 0.20 " = 18.5 " Match perfect. **93.0 %**	0.10 cc. = 7.6 mm. 0.20 " = 15.2 " Match perfect. **76.0 %**	1.4 cc. N_2 at 22° and 745 mm. 0.1575 gm. histidine Cl_2. **78.8 %**	0
2	10 cc. 2 % histidine Cl_2. 100 cc. Nutritive Medium 3. 90 cc. distilled water. 3.8 gm. Na_2HPO_4. 12 H_2O.	0.10 cc. = 8.6 mm. 0.20 " = 17.2 " Match perfect. **86.0 %**	0.10 cc. = 8.3 mm. 0.20 " = 16.7 " Match perfect. **83.0 %**	1.5 cc. N_2 at 26° and 754 mm. 0.167 gm. histidine Cl_2. **83.5 %**	0
3	10 cc. 2 % histidine Cl_2. 100 cc. Nutritive Medium 3. 90 cc. distilled water. 0.5 gm. sodium formate.	0.10 cc. = 10.0 mm. 0.20 " = 20.0 " Match perfect. **100.0 %**	0.10 cc. = 9.2 mm. 0.20 " = 18.2 " Match perfect. **92.0 %**	1.73 cc. N_2 at 27° and 747 mm. 0.1895 gm. histidine Cl_2. **95.0 %**	0
4	10 cc. 2 % histidine Cl_2. 100 cc. Nutritive Medium 3. 90 cc. distilled water. 1 cc. 2.5 % filtered litmus solution.†	0.10 cc. = 10.0 mm. 0.20 " = 20.0 " Match perfect. **100.0 %**	0.10 cc. = 9.8 mm. 0.20 " = 19.7 " Match perfect. **98.0 %**	1.77 cc. N_2 at 26° and 754 mm. 0.1974 gm. histidine Cl_2. **98.7 %**	0

* Color matched against the (CR–MO) standard.

† See special record, page 580.

This is probably high by 0.6 per cent due to admixture with histidine.

I.

sults. Part III.

'olor value of histamine fraction.*	Histidine converted into histamine (colorimetric determination).	Histidine converted into methyl imidazole (Methods B and C).	Amount of 0.1 N HCl neutralized by ammonia from test solution.	Total N as NH_3 (N_2 in KNO_3 method).	Histidine converted into histamine (Van Slyke method) with 5 cc. of test solution.	Reaction: Before incubation.	Reaction: After incubation.
		per cent	*cc.*	*per cent*		*pH*	*pH*
.10 cc. = 5.0 mm. .50 " = 25.1 " latch perfect; identical with that obtained with histamine.	0.0165 gm. histamine in test solution. 10.2 % of histamine present.	0	23.0	36.7	0.22 cc. N_2 at 22° and 750 mm. 0.02011 gm. histamine Cl_2. 12.5 %	7.5	> 6.6
50 cc. = 10.2 mm. 0 " = 20.2 " latch perfect; identical with that obtained with histamine.	0.0068 gm. histamine in test solution. 4.2 % of histamine present.	0	23.5	37.5	0.06 cc. N_2 at 30 and 752 mm. 0.00527 gm. histamine Cl_2. 3.25 %	7.7	Slighlty >6.6
0 cc. = 9.7 mm. atch good for histamine.	0.00325 gm. histamine in test solution. 2.0 % of histamine present.	0	34.7	55.3		7.4	6.6
0 cc. = 5.7 mm. atch good; more like histidine than histamine.	0.00175 gm. histamine in test solution. 1.08 %‡ of histamine present.	0	18.5	29.5	0.02 cc. N_2 at 30 and 752 mm. 0.001758 gm. histamine Cl_2. 1.09 %	Medium kept approximately neutral to litmus by addition of N NaOH.†	

much more efficient acid neutralizer sodium carbonate. In this case then, only 2 per cent of the histidine was converted into histamine.

SUMMARY.

1. When the colon bacillus is allowed to metabolize histidine, either alone or in the presence of nitrates or ammonium salts, histamine is not formed.

2. In a medium containing histidine and glycerol, but no nitrates or ammonium salts, histamine is not formed. In this case imidazole propionic acid appears to be formed; but only when the bacillus is forced to grow anaerobically.

3. In a medium containing histidine, glycerol, or glucose and a source of nitrogen, either KNO_3, NH_4Cl, or both, about 50 per cent of the histidine is converted into histamine in the course of 2 weeks when oxygen is present. In the absence of atmospheric oxygen, this and all the other metabolic activities of the bacillus are greatly reduced, probably because the colon bacillus is an aerobic organism by preference.

4. The production of histamine is always coincident with the production of a medium that is distinctly acid. We believe that the histamine is formed by the bacillus to neutralize the excess of acidity that is simultaneously produced from the glycerol.

5. Contrary to the statement sometimes given in text-books and in the literature that carbohydrates prevent the formation of histamine from histidine, we have found that histamine is never formed except in the presence of an easily available source of carbon such as glycerol or glucose.

Reprinted from THE JOURNAL OF BIOLOGICAL CHEMISTRY, Vol. XXXIX, No. 3, 1919

STUDIES ON PROTEINOGENOUS AMINES.

V. THE PREPARATION OF P-HYDROXYPHENYLETHYLAMINE HYDROCHLORIDE (TYRAMINE HYDROCHLORIDE).

By KARL K. KOESSLER AND MILTON T. HANKE.

(*From the Otho S. A. Sprague Memorial Institute and the Department of Pathology, University of Chicago.*)

(Received for publication, July 14, 1919.)

The synthesis of *p*-hydroxyphenylethylamine reported herewith is based on the synthesis of *p*-hydroxybenzylcyanide reported by Pschorr, Wolfes, and Buckow[1] and on its reduction as reported by Barger.[2] Benzylcyanide is nitrated, *p*-aminobenzylcyanide obtained by reduction of the nitro compound, and the *p*-hydroxybenzylcyanide, obtained with the aid of nitrous acid, is then reduced with sodium and alcohol. The improvements that we have to suggest on the first three steps are largely mechanical. The preparation and purification of the final product, *p*-hydroxyphenylethylamine, was carried out according to the scheme previously developed for histamine[3] which is rather more efficient than the method reported by Barger.

The nitration of benzylcyanide offers no difficulties when the method of Pschorr, Wolfes, and Buckow[1] is carefully followed. It is necessary to purify the product thoroughly by recrystallization from alcohol before proceeding to the next preparation. An impure product gives rise to a highly colored amino compound on reduction which is hard to purify.

Preparation of p-Aminobenzylcyanide.

In the preparation of *p*-aminobenzylcyanide, the following further suggestions may be of value.

[1] Pschorr, R., Wolfes, O., and Buckow, W., *Ber. chem. Ges.*, 1900, xxxiii, 170.

[2] Barger, G., *J. Chem. Soc.*, 1909, xcv, 1127.

[3] Koessler, K. K., and Hanke, M. T., *J. Am. Chem. Soc.*, 1918, xl, 1722.

After the reduction is complete and the alcohol has been removed by distillation *in vacuo* at 40°[1] the mixture is cooled in an ice bath, covered with a layer of 250 cc. of ether, and treated with a solution of 70 gm. of sodium hydroxide in 150 cc. of water, the alkali being added very slowly so that the temperature of the mixture does not rise above 20°. After all the alkali has been added, two sharply defined layers are obtained, the lower being slightly grey due to precipitated tin, the upper being very pale yellow. The ether layer is decanted as completely as possible, and the aqueous alkaline layer reextracted four times with ether, 200 cc being used for each extraction. The combined ether extracts, which should be nearly colorless, are dried rapidly over anhydrous sodium carbonate and the ether removed by distillation, at first at ordinary pressure and then *in vacuo*. An entirely crystalline, pale yellow solid was usually obtained, with a melting point of 44–46° and whose weight was from 95 to 98 per cent of that demanded by the theory. If a dark-colored product is obtained, which will never be the case when all the precautions given above are observed, this can be readily purified by a vacuum distillation. This gives a pale green liquid, whose boiling point is 198° at a pressure of 10 mm., which solidifies readily in the ice box to a pale greenish white solid that soon becomes almost colorless and melts at 45–46°. The product can be kept indefinitely in a tightly stoppered bottle at ice box temperature. At room temperature it gradually becomes brown and sticky.

Preparation of p-Hydroxybenzylcyanide.

Although the description given by Pschorr, Wolfes, and Buckow of the preparation of *p*-hydroxybenzylcyanide leads one to believe that they had the details carefully worked out, they do not report them completely enough to make an exact repetition possible. We take the liberty, therefore, to report the details of our experiments.

A solution of 20 cc. of 95 per cent sulfuric acid in 200 cc. of water is heated to boiling over an asbestos gauze in a 1,000 cc. Florence flask. 6.6 gm. of *p*-aminobenzylcyanide—0.05 mol—is then added to the gently boiling solution. The locally precipitated sulfate of the base dissolves rapidly in the hot solution. The

flame is turned low so that the liquid is kept just below the boiling point. The flask is then equipped with a three-hole rubber stopper into which are inserted a thermometer and a dropping funnel, both of which extend to within a few mm. of the bottom of the flask. A solution of 4 gm. of 90 per cent sodium nitrite in 40 cc. of water is allowed to flow steadily under the acid liquid in the course of 15 minutes. The temperature of the liquid should remain between 95–100°. The liquid appears to boil due to the steady evolution of nitrogen which should contain little or no nitrous anhydride. 50 cc. of water are then added to the reaction liquid, and the contents of the flask heated to boiling.

The solution so obtained, because of its high acid content, is free from the purple dye that is always formed if too little acid is present. A very little dark brown tar is formed. To remove this, the *hot* liquid is treated with about 5 gm of animal charcoal and filtered into a 1,000 cc. flask. The yellow filtrate is cooled in tap water. A colorless oil is deposited which is *p*-hydroxybenzylcyanide. On long standing, or quickly after seeding, the oil solidifies, so a primary crop of crystals could be obtained in this way. It is not advisable to separate this material at this time, however, because an ether extraction of the acid liquid is necessary under any condition. The cold, cloudy, yellow, acid liquid is, therefore, extracted four times in the flask with ether, using about 200 cc. of ether for each extraction. (The first extract contains practically all the *p*-hydroxybenzylcyanide.) The pale yellow ether solution is then extracted twice with 25 cc. of a saturated aqueous solution of bicarbonate of soda, and once with 25 cc. of water. The bicarbonate solution removes all the *p*-hydroxyphenylacetic acid and nearly all the coloring matter. The ether is then removed by distillation, at first under ordinary pressure and then *in vacuo*. There is thus obtained 5.5 gm. of nearly pure substance in the form of a pale yellow crystal cake. This is 83 per cent of that required by theory.

The substance so obtained can be further purified by distillation *in vacuo*—it boils at 210° at 10 mm. pressure—or it can be recrystallized from boiling water. The crystal cake is dissolved in 10 parts of boiling water, the solution clarified with a little animal charcoal after adding a few drops of 37 per cent hydrochloric acid, and filtered. On cooling and seeding the filtrate

deposits a colorless oil which rapidly becomes entirely crystalline. The white crystals weigh 75 per cent of the starting material and melt at 67–71°. To obtain a second crop it is advisable to extract the mother liquor with ether and extract the ether with bicarbonate solution as above.

Preparation of p-Hydroxyphenylethylamine Hydrochloride (Tyramine Hydrochloride).

Barger prepared the free base of the above substance by a process that was laborious and time-consuming. He made no attempt to isolate the by-products. The method outlined below gives all the products that are formed in the reaction in a fairly high state of purity, the tyramine being obtained in its most desirable form, the hydrochloride.

5 gm. of *p*-hydroxybenzylcyanide, melting point 168–171°, were treated with 50 cc. of absolute alcohol in a 500 cc. round bottomed flask. The flask was suspended from a Liebig condenser by means of a rubber stopper, and the alcohol heated to boiling by means of a small flame which impinged against an asbestos gauze. A $\frac{1}{2}$ inch air space between the gauze and the bottom of the flask prevented superheating and charring of the sodium ethylate which precipitated in the course of the reaction. 10 gm. of metallic sodium, about two equivalents, were rapidly added through the condenser in small pieces, the time of addition being 5 minutes. After boiling for $\frac{1}{2}$ hour, 20 cc. of absolute alcohol were added through the condenser to facilitate the solution of the sodium, and to dissolve the precipitated sodium ethylate. It was necessary to add 20 cc. more of absolute alcohol after 45 minutes of total heating, to dissolve all the sodium.

After the metal had completely dissolved, the mixture was allowed to cool somewhat. 100 cc. of water were then added to the warm mixture. The resulting clear, yellow solution was freed from alcohol, ammonia, and some water by distillation *in vacuo* to a volume of about 50 cc. Enough concentrated hydrochloric acid was then added to give a strongly acid reaction to litmus, the flask and contents being cooled in tap water while the acid was being added. The resulting pale yellow solution was transferred with a little water to a graduated separatory funnel.

Its final volume was about 150 cc. A small amount of oil, *p*-cresol, floated on the aqueous liquid.

Extraction of p-Cresol and p-Hydroxyphenylacetic Acid.

The solution was freed from the above two compounds by extracting it five times with ether, using 200 cc. of ether for each extraction. The further treatment of this ether extract will be discussed below.

Extraction of Tyramine.—The liquid so obtained was rendered strongly alkaline by the addition of anhydrous sodium carbonate and extracted exhaustively with amyl alcohol using 150 cc. of the alcohol for each extraction. From seven to ten extractions were necessary to remove the tyramine completely. The aqueous layer gave only a pale pink Pauly reaction when the extraction was complete. It was discarded.

The combined amyl alcohol extracts were dried over anhydrous sodium carbonate, filtered from the carbonate using a Buchner funnel, and the clear, pale brown filtrate was extracted three times with 1.0 N HCl and seven times with water using 100 cc. for each extraction.

The combined aqueous acid extracts were freed from water and hydrochloric acid by distillation *in vacuo* at 50°. A voluminous, pale yellow solid was left in the flask which weighed 3.8 gm. and which was nearly pure tyramine hydrochloride. This is 58 per cent of that required by theory.

The solid obtained from three experiments, 11.1 gm., was treated with 5 cc. of concentrated hydrochloric acid and 100 cc. of absolute alcohol. By heating on the water bath practically all the solid was brought into solution. The liquid was filtered from a slight residue of sodium chloride. On cooling the filtrate, tyramine hydrochloride crystallized out in the form of fluffy, glistening needles. The mixture was filtered with suction and the crystals were washed freely with absolute alcohol. Dried *in vacuo* for 48 hours over sulfuric acid, the pure white solid weighed 9.1 gm. and had the following properties.

1. It melted to a clear brown liquid at 280° (Corrected).
2. It was entirely free from ammonium chloride.
3. It gave an intense orange red color reaction with paraphenyl-diazonium sulfonate.

4. It left a residue on ignition at dull red heat of 1.86 per cent; *i.e.*, 0.3000 gm. ignited in a platinum crucible left a residue of 0.0056 gm., which proved to be sodium chloride. This would suggest that the product was 98.14 per cent pure.

5. A Van Slyke amino nitrogen determination gave the following results. 0.0100 gm. of substance gave 1.48 cc. of nitrogen gas at 28° and 749 mm. If the material was 98.14 per cent pure, the actual weight of pure tyramine hydrochloride used was 0.009814 gm.

	Calculated.	Found.
Per cent amino nitrogen	8.06	8.11

6. A chlorine determination gave the following results. 0.2000 gm. of solid took 11.87 cc. of 0.1 N $AgNO_3$. If the solid is considered to be 98.14 per cent tyramine hydrochloride and 1.86 per cent sodium chloride, 11.93 cc. should have been used.

7. A solution containing 0.002 gm. of substance was injected into the femoral vein of a dog, weighing 4.7 kilos, that had been previously anesthetized and prepared so that a blood pressure tracing could be obtained. The tracing obtained, Fig. 1, showed that the typical rise in blood pressure had been produced by the substance.

This series of seven tests proves conclusively that the substance was *p*-hydroxyphenylethylamine. Its purity was 98.14 per cent. The remaining 1.86 per cent consisted entirely of sodium chloride.

Separation of p-Cresol from p-Hydroxyphenylacetic Acid.—The combined ether extracts from five experiments were concentrated to a volume of about 1,000 cc. The ether was extracted five times with a 10 per cent sodium carbonate solution using 40 cc. for each extraction. The combined carbonate solutions were then extracted five times with ether using 100 cc. for each extraction. This divided the material into two fractions; the ether fraction which contained all the *p*-cresol and was free from *p*-hydroxyphenylacetic acid, and the alkaline aqueous fraction which was free from cresol but contained all the *p*-hydroxyphenylacetic acid.

Separation of Pure p-Cresol from the Ether Fraction.—The ether extracts were freed from ether by distillation first at ordinary pressure and then *in vacuo*. There were thus obtained 5.5 gm. of pale brown oil having the odor that is characteristic of *p*-cresol. This oil distilled over completely at a temperature of 198–200° at

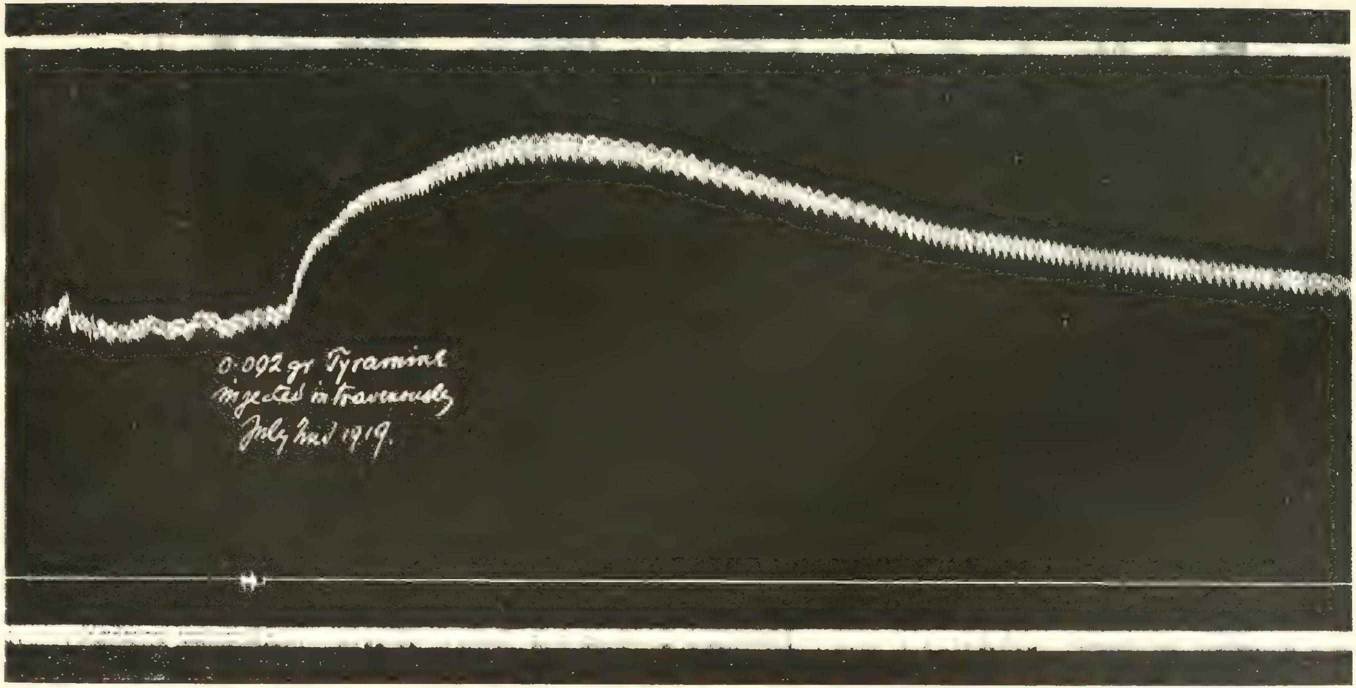


FIG. 1. Respiratory and blood pressure tracing obtained by injecting 2.0 mg. of synthetic tyramine hydrochloride into the femoral vein of a dog.

ordinary pressure and boiled at 90–91° at a pressure of 10 mm. The perfectly colorless oil obtained was pure *p*-cresol. (The recorded boiling point of *p*-cresol is 198°.)

Separation of Pure p-Hydroxyphenylacetic Acid.—The alkaline aqueous liquid was treated with an excess of hydrochloric acid and extracted with ether until the ether extracts no longer gave a Pauly reaction. The ether was then dried over calcium chloride and distilled at first under ordinary pressure and then *in vacuo*. There were thus obained 2.3 gm. of a pale yellow solid which was dissolved in water, the solution decolorized with charcoal, and redistilled *in vacuo*. The resulting white solid was dissolved in 4 cc. of hot water. 8 cc. of concentrated hydrochloric acid were then added drop by drop. This caused the *p*-hydroxyphenylacetic acid to precipitate in large, white, flat, prismatic needles melting sharply at 150° (Corrected). 1.7 gm. of the perfectly pure product was obtained. The recorded melting point of *p*-hydroxyphenylacetic acid is 148° (Uncorrected).

[Reprinted from the Journal of Industrial and Engineering Chemistry, Vol. 11, No. 9, page 893. September, 1919.]

EXPLOSIONS WITH AMMONIACAL SILVER OXIDE SOLUTIONS

Editor of the Journal of Industrial and Engineering Chemistry:

Considering the frequency with which the solubility of silver oxide and silver chloride in ammonium hydroxide is utilized in the chemical laboratory, especially in undergraduate courses, the fact that silver fulminate is readily obtained by the treatment of silver oxide with ammonium hydroxide is not sufficiently well known. It is the purpose of this note to warn of the danger incurred when *concentrated ammonium hydroxide reacts with silver oxide* and to urge that a warning be placed in all procedures in which ammoniacal silver oxide solutions are used.

My own experiences on the formation of explosive silver fulminate occurred some years ago while studying the oxidation of some organic compounds with silver oxide. In order to determine the amount of oxygen consumed the undissolved residue ($Ag + Ag_2O$) was treated with ammonium hydroxide to dissolve the unchanged silver oxide. The silver residue was then dried and weighed. On one occasion such a precipitate mysteriously disappeared overnight from a hot plate (on which it had been left to dry) along with much glass apparatus. Later, evidence was obtained to show that the apparatus was fragmented and cleanly swept away by the explosion of the precipitate.

On another occasion a flash of flame and a charring of the filter paper was observed when something gently exploded while washing a precipitate with concentrated ammonium hydroxide.

On the last occasion the contents of a small beaker containing ammoniacal silver oxide exploded just after stirring the precipitate with a small, glass rod. It was noticed that gas bubbles were evolved just a moment before the liquid exploded. About 200 minute fragments of glass were driven into my forearm and the strongly alkaline liquid was thrown into my eyes. I was fortunately only temporarily inconvenienced. That the power of this explosion was terrific is indicated by the fact that some of the small glass fragments (less than 1 mm. in diameter) were driven through the walls of flasks and condensers as much as 10 ft. away. These holes were so small that some of them were not detected for months afterwards.

Raschig[1] who fully investigated silver fulminate obtained in this way states that the violence of the explosion is much overestimated. He, however, was careful to handle his product in very small portions. Larger amounts would have impressed him more.

Matignon[2] observed an explosion of an ammoniacal silver oxide solution that stood overnight, which suggests that such solutions should not be kept without proper precautions.

Raschig states that explosive derivatives are never formed with dilute ammonia and silver oxide. My own experience confirms this.

For further details the two papers referred to should be consulted.

E. J. WITZEMANN

OTHO S. A. SPRAGUE MEMORIAL INSTITUTE
CHICAGO, ILLINOIS
March 25, 1919

[1] *Ann.*, **233** (1886), 93.

[2] *Bull. Soc. Chim.*, [4] **3** (1908); *Chem.-Ztg.*, **32**, 607; *Chem. Zentr.*, [II] **1908**, 136.

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[CONTRIBUTION FROM THE OTHO S. A. SPRAGUE MEMORIAL INSTITUTE, RUSH MEDICAL COLLEGE.]

THE VOLATILITY WITH STEAM OF LOWER FATTY ACIDS IN DILUTE AQUEOUS SOLUTIONS.

BY EDGAR J. WITZEMANN.

Received August 4, 1919.

In some tests of the volatility of the lower α-hydroxy aliphatic acids in dilute aqueous solutions of formic, acetic, propionic or butyric acids, it was again observed[1] that the behavior of the fatty acids in this respect is contrary to the laws of simple mixtures. The 4 fatty acids in dilute aqueous solutions show an increasing volatility with increasing molecular weight. It is proposed here to give briefly a few experimental data and to indicate how these relationships, which are commonly stated to be abnormal, are to be expected on the basis of existing knowledge of the molecular state and hydration of these acids in aqueous solution.

The experiments were done as follows: 2 g. of formic acid (by titration) and the corresponding molecular amounts of the 3 other acids, were each made up to 200 cc. with water. Five cc. of the solutions were pipetted off and titrated with 0.1 N sodium hydroxide solution in order to confirm the concentration of the solution. The remaining 195 cc. was transferred to a common distilling flask (500 cc.) and distilled off slowly in 20 cc. fractions correct to within one drop. The receiver (a calibrated test-tube) was emptied and rinsed. The fractions were then titrated separately with 0.1 N sodium hydroxide solution. Typical data are given in the table.

TABLE I.

Relative Volatility of Lower Fatty Acids. (Cc. 0.1 N NaOH).

	Fraction. Cc.	Formic. Cc.	Acetic. Cc.	Propionic. Cc.	Butyric. Cc.
	5	10.80	10.82	10.78	10.81
1	20	16.70	29.33	52.87	81.92
2	20	19.07	30.85	52.10	71.35
3	20	20.36	32.36	50.10	62.98
4	20	22.65	33.80	47.94	53.77
5	20	24.40	35.63	45.60	44.62
6	20	28.48	38.40	43.00	36.30
7	20	33.76	41.45	39.90	28.39
8	20	42.17	46.70	36.60	20.34
9	20	59.45	56.00	33.03	13.36
Residue		155.88	79.05	24.40	6.58
Total		422.92	423.57	424.54	419.61
Calculated		421.20	421.98	420.42	421.59

The above results are given graphically in Fig. 1.

It appears clearly that there is a gradually increasing volatility of

[1] Duclaux, *Ann. chim. phys.*, **2**, 289 (1874); *Ann. inst. Pasteur*, **9**, 265 (1895); cf. also Upson, Plum and Schott, THIS JOURNAL, **39**, 731–42 (1917).

these 4 acids of the aliphatic series with increasing molecular weight, although the boiling points of the acids rise gradually from 101° for formic to 162° for butyric acid. Thus the first fractions contain 3.94% of the formic, 6.92% of the acetic, 12.4% of the propionic, and 19.5% of the

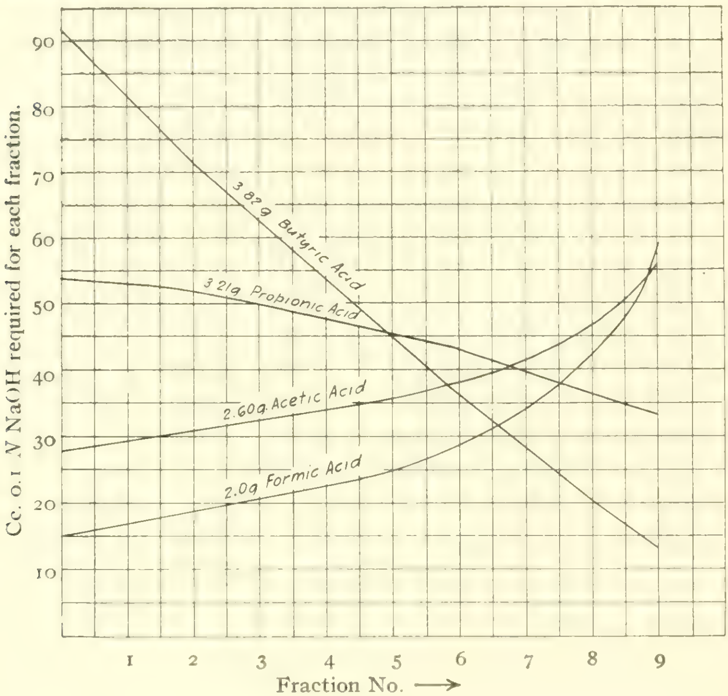

Fig. 1.—Relative volatility of the lower fatty acids from boiling aqueous solutions.

butyric acid used, respectively. For the last fractions this order is reversed as shown, and the acid remaining in the distilling flask is as follows: formic acid 36.8%, acetic 18.6%, propionic 5.7%, butyric 1.4%, respectively, of the acid taken.

The interpretation of these data on the basis of existing knowledge may be briefly stated as follows:

(1) That along with their clearly demonstrated powers of molecular association[1] these 4 acids form hydrates with water.

(2) The hydration, as well as the molecular association of these acids, varies in amount and stability.[2]

[1] Jones, *Carnegie Inst. Publications*, **1915**, 210; cf. summary and list of earlier publications at the end. Turner, "Molecular Association," Longmans, Green & Co. (1915).

[2] Cf. Turner, *Loc. cit.* for review; Roscoe, *Ann.*, **125**, 320 (1863); Peddle and Turner, *J. Chem. Soc.*, **89**, 1439 (1907); Colles, *Ibid.*, **89**, 1246 (1906); Jones, *Loc. cit.*, p. 148.

(3) The hydrates of greatest stability appear to occur with formic acid (cf. Roscoe, Colles) and this stability appears to diminish with increase in the molecular weight of the 4 fatty acids. It seems evident that this greater stability is due to affinities of the carboxyl group and that the diminishing stability is associated with the increasing hydrocarbon function.[1]

(4) That the hydrates of these acids presumably have the power of lowering the partial vapor pressure of the acid in dilute solutions was definitely established, at least for formic acid, by Roscoe.[2]

(5) It is because of this capacity of the hydrates to lower the partial vapor pressure of the acid that the volatility of these acids in dilute aqueous solution presents a reverse picture as compared with what would be expected if the solutions were simple physical mixtures of the two ingredients. When the decreasing stability of the hydrates at the boiling point of the mixture is considered it appears that the behavior of these acids from the standpoint of simple mixtures tends to become less anomalous on passing from formic to butyric acid. Accordingly, hydration phenomena probably have less influence on the volatilization of higher fatty acids (stearic acid) with steam than on those here discussed.

(6) The behavior of dilute solutions of these acids on distillation apparently constitutes evidence of hydration of these compounds since it agrees with all the other data on this subject.

Of the above statements (5) and (6) are perhaps novel.

The Effect of Neutral Salts.

From the existing data regarding the effects of salts on the hydration of another salt in the same solution[3] as well as from Kolossovsky's[4] observations of the influence of salts on the partition of acetic acid between ether and water, the addition of a neutral salt, to such solutions as were used above should produce an increase in the volatility of the fatty acid. This increase in volatility should be greater the greater the concentration of the salt used. That this is true is shown by the data represented in Fig. 2, which were obtained by the same method used above, except that the acetic and formic acids used were weighed. In a 0.5 M solution of magnesium chloride (III) the 15 cc. of residue remaining in the distilling flask contains no acetic acid. In (II) this residue consumes 22.65 cc. of 0.1 N sodium hydroxide solution, and in (I) it requires 55.75 cc.

[1] This statement is also supported by Auwer's generalizations on the relation of constitution of solvent and solute to the molecular weight of the latter (*Z. physik. Chem.*, **15**, 33 (1894); **18**, 595 (1895); **30**, 529 (1899); **42**, 513, 542 (1903)) provided the reasonable assumption is made that the ethyl and propyl groups come first beyond the methyl group in this series.

[2] *Loc. cit.*

[3] Jones and Stine, *Am. Chem. J.*, **39**, 313 (1908).

[4] *Bull. Soc. chim. belg.*, **25**, 183 (1911); *Bull. Soc. chim.*, [4] **9**, 632 (1911).

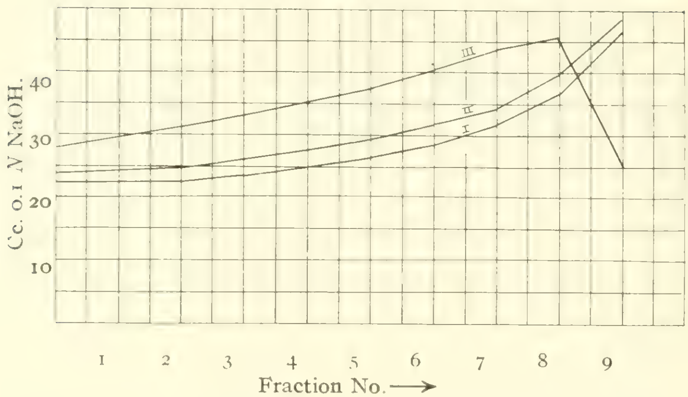

Fig. 2.—Effect of dissolved magnesium chloride on the volatility of acetic acid from boiling aqueous solutions.

2.0 g. acetic acid in 200 cc. { I—H_2O. II—0.1 *M* $MgCl_2$. III—0.5 *M* $MgCl_2$.

In Fig. 3 similar results for formic acid are given.

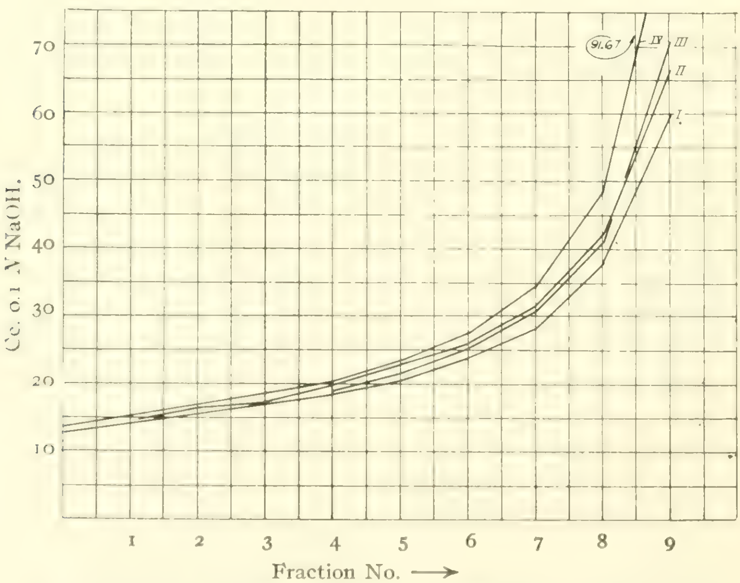

Fig. 3.—Effect of dissolved potassium and magnesium chlorides on the volatility of formic acid from boiling aqueous solutions.

2 g. formic acid in 200 cc. { I—H_2O. II—0.1 *M* $MgCl_2$. III—0.5 *M* KCl. IV—0.25 *M* $MgCl_2$.

That this increased volatility is not solely a function of the concentration of the salt is suggested by the results for (II) and (III) in Fig. 3. For (III), in which the molecular salt concentration is 5 times greater than in (II), the increase in volatility over (I) averages less than for (II). This apparently indicates that 5 molecules of potassium chloride have a little less positive influence on the volatility of formic acid in this solution than one molecule of magnesium chloride. In experiments with acetic acid, not described here, in a 0.5 M potassium chloride solution, the volatility was just a little greater than in a 0.1 M magnesium chloride solution.

These results are relatively just what would be expected from our knowledge of the hydration of these salts and thus give further confirmation to the interpretation of the volatility of these acids given above.

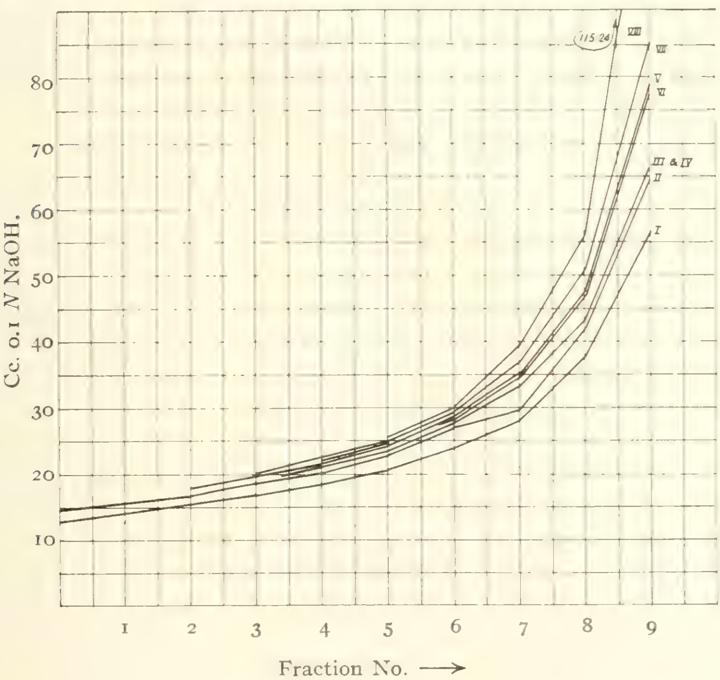

Fig. 4.—Influence of dissolved salts (0.25 M) on volatility.

2.0 g. formic acid in 200 cc.:
- I—H_2O.
- II—KCl.
- III—NaCl sol.
- IV—$BaCl_2$ sol.
- V—$SrCl_2$.
- VI—$CaCl_2$.
- VII—$MgCl_2$.
- VIII—$AlCl_3$.

It was of interest to learn whether these relations could be extended in the manner suggested by the deductions of Poma and Albonico[1] regarding the neutral salt effect on the hydrolysis of esters. If their deductions were applicable here equimolecular amounts of metallic salts such as chlorides, for instance, should produce an increasing volatility with the decreasing electro-affinity of the metallic ion of the added salt. Experiments with 0.25 *M* solution of the chlorides of potassium, sodium, barium, strontium, calcium, magnesium, aluminum, manganese, iron and copper were carried out. The volatility was, slightly and progressively, a little increased for each member of this series over the preceding member up to and including aluminum, except in the case of strontium and calcium, for which the order given above should be reversed. The solutions of manganese, iron and copper chlorides showed anomalies and give increases in volatilities lower than aluminum chloride.

Part of the results are given in graphic form in Fig. 4. The results for sodium and barium chlorides are so nearly alike that the results for sodium chloride only were plotted (Curves III and IV).

CHICAGO, ILL.

[1] *Atti accad. Lincei,* **24,** I, 747, 979; II, 43 (1915).

FEVER AND THE WATER RESERVE OF THE BODY

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INTRODUCTION

During the course of continued intravenous injections of glucose in dogs[1] and man[2] described elsewhere, fever was observed under certain conditions, namely, when the rate of sugar injection was sufficiently in excess of the tolerance limit to produce a marked glycosuria with its concomitant diuresis; when the rate of water administration was less than the rate of diuresis and when these conditions were sustained until the animal or man had lost a certain weight by dehydration. Chills were also observed to occur under the same conditions after the body temperature had begun to rise. Both chills and fever were seen to subside when enough additional water was administered. In the experiments on dogs, the rates of sugar administration were controlled by one motor driven pump, the rates of water injection by another pump. The urine was collected and measured continuously by a catheter retained in the bladder which emptied into a graduated cylinder. The animals lay constantly on the platform of a scales, sensitive to 10 grams. Under these conditions the water balance was subject to absolute control and gains or losses of 10 grams or more in body weight were readily detectable. In this way, fever and chills could be made to come and go at will during the course of a single experiment.

The most obvious explanation was that after a sufficient quantity of water had been abstracted from the body, there was not enough left to sustain the normal processes of cooling by evaporation through the lungs (and the skin in the case of man), and that the animals suffered a true thirst fever produced in an unusual way. The view was also expressed that the actual removal of water from the body might not be essential and that the mere presence of a sufficient quantity of extra

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* From the Otho S. A. Sprague Memorial Institute Laboratory for Clinical Research, Rush Medical College.

1. Woodyatt, R. T.: Studies in Intermediate Carbohydrate Metabolism. Harvey Lectures, 1915-16, p. 326.

2. Sansum, W. D.: Rapid Reduction of Intraocular Tension in Glaucoma. J. A. M. A. **68**: 1885 (June 23), 1917.

sugar in the blood and tissues might serve to bind water in a physico-chemical union which would render it unavailable for evaporation at the normal body temperature.

However, various other explanations could be formulated. It might be held that during the rapid injections of glucose an increased oxidation of sugar contributed to the end result, or that the process disturbed a nervous heat regulating "center" or that the sugar set up changes of one type or another in the cells, with fever as a secondary consequence. As a matter fact, the literature of sugar and salt fevers, beginning with Finkelstein[3] in 1908, contains discussions of all these possibilities without reaching a common decision. The literature is clear, however, in showing that sugar and salt fevers disappear when enough water is administered resembling in this respect "inanition" fever of the new-born, which as Crandell[4] showed is due to thirst. The later writers on salt fever, Heim and John[5] and Peteri,[6] clearly interpret salt fever as a result of decreased evaporation of water due to the hydropigenous (or edema producing) action of salt (sodium chlorid) in the blood and tissues. Nearly all of the literature on this subject has been contributed by the pediatricians and the fevers in question have usually followed single doses of salt or sugar by the alimentary or subcutaneous route in infants. Although fever following single intravenous injections of sugar in adults[7] had been reported before our own experiments, there was, on the other hand, enough difference between the methods of producing salt and sugar fever as described in the pediatric literature and that which we describe that at first it was not clear that we were dealing with the same phenomenon. Early failure on our part to confirm the production of fever by single subcutaneous, alimentary or intravenous administrations of sugar in adult dogs or man, suggested that we were not. But it was later found that this could be done consistently in dogs if the animals were made sufficiently thirsty beforehand. The uniform success of certain pediatricians in producing salt fever in babies suggests that they also have worked regularly with thirsty animals.

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3. Finkelstein, H.: Ueber Alimentäre Intoxikation. Jahrb. f. Kinderh. **68**: 693 (Dec.), 1908.

4. Crandell, F.: Inanition Fever. Arch. Pediat. **16**: 174 (March), 1899.

5. Heim, P. and John K.: Pyrogene und hydropygene Eigenschaften der Physiologischen Salzlösung. Die Bedeutung und Behandlung Exsiccation. Arch. f. Kinderh. **54**: 65, 1910.

6. Peteri, I.: Beiträge zum Pathologischen Wesen und zur Therapie des Transitorischen Fiebers bei Neugebornen. Jahr. f. Kinderh. **80**: 612 (Dec.), 1914.

7. Bingel, A.: Ueber Salz- und Zucker-Fieber. Arch. f. exper. Path. u. Pharmakol. **64**: 1, 1910.

Our own experiments appeared to offer a very good method of producing and allaying fever in the laboratory under conditions more susceptible to control than any method heretofore used, and it was thought advisable to test its limits as a method. The experiments herein described were undertaken primarily for that purpose. It was also thought desirable to employ the method to investigate further into the mechanism of this type of fever and chills in the hope that light might be thrown on the mechanism and possibly the rational management of fevers and chills in general, particularly the toxic fevers, such as typhoid, the mechanism of which has not yet been settled. Several points bearing on these questions are elaborated in the discussion. The question presents itself as to whether all ordinary clinical fevers are not due in the last analysis to a deficit of free water in the body.

EXPERIMENTS

These were designed to determine:

1. How high the temperature of the body can be driven by the technic in question.

2. Whether or not an increased combustion of glucose is a contributing factor.

3. Whether the fever is due to a primary effect of the sugar on the water of the body or whether it is secondary and dependent in any way on a nervous "center" or nervous heat regulating mechanism.

4. Whether it is necessary actually to remove water from the body or whether molecules of sugar within the body can produce fever by their mere presence.

The experiments show that the body temperature can be driven to remarkable heights by this method. Fevers of 111 F. are readily produced, and in one case the body temperature rose to 125.6 F. Fevers of 111 F. were produced by means of salt (NaCl) and by lactose, showing that the increased combustion of glucose is not a necessary factor, which was the conclusion to be anticipated in view of the fact that diminished heat loss and not increased heat production (or supply) is known to be the determining factor in all fevers, that is to say, a great increase of heat production alone does not cause fever because normally a great excess of heat is disposed of automatically by increased cooling. The experiments show that sugar is capable of causing fever by its mere presence in the body under certain circumstances, but that no quantity of sugar which can easily be introduced will cause fever unless the water reserve of the body is for some reason sufficiently low to begin with. Finally, sugar fever can be

produced in dogs which have been rendered poikilothermic by severing the cervical cord, and therefore these fevers are in no sense dependent on a nervous heat regulating mechanism.

Experiment 1. — A female bull-terrier, weighing 9.3 kilos, was injected intravenously, with a 36 per cent. glucose solution, at the rate of 15 gms. of glucose per kilogram of body weight per hour, for a period of ninety minutes. During this time the animal received 614 c.c. of the glucose solution (containing 221 gms. of glucose) and passed 1,230 c.c. of urine, containing 68.4 gms. of glucose; a retention of 152.6 gms. The fluid output exceeded the intake by 616 c.c., or 66.2 c.c. per kilo. The temperature (vaginal), which was 102 F. at the beginning of the experiment, increased to 102.5 F. by the end of the first hour. A severe chill began twenty-one minutes later and the dog was covered with a woolen blanket. Nine minutes later violent convulsions began and the temperature was found to be 111.2 F. The convulsions became so violent that it was necessary to stop the injection. The urine ceased to flow and thirteen minutes later the dog died with a temperature of 125.6 F., the highest animal temperature that we have been able to find on record. The rise of temperature after the cessation of diuresis amounted to 14.4 degrees.

A postmortem examination was made immediately after death in the presence of Prof. H. G. Wells. The blood was turbid. The red blood cells were crenated to a marked degree. The heart was uniformly dilated to the limit of the pericardial sac. The kidneys and liver were in a state of "cloudy swelling." (Note that in spite of the loss of 66.2 c.c. of water for each kilogram of body weight, with crenation of the red cells, the parenchymas of the liver and kidneys, though dry to the touch, had the appearance of "cloudy swelling.")

Experiment 2.—The course of this experiment was similar to that of the above, except that no covering was thrown over the animal at the onset of the chills and the diuresis and temperature rise were not so marked.

The dog, weighing 16.9 kilogram, received a 36 per cent. glucose solution, at the rate of 15 gm. per kilogram per hour, for ninety minutes. During this time the fluid intake was 1,029 c.c. and the urinary output 1,470 c.c., a dehydration of 441 c.c. or 27.8 c.c. per kilogram. The temperature, which was 100.4 F. at the beginning of the experiment, rose to 109.4 F.

In previous experiments with glucose injections at the rate of 3.6 gm. per kilogram per hour it was shown that only about 0.6 gm. per kilogram is actually burned. However, the amount of combustion might increase somewhat with the higher rate of injection. Accordingly, increased combustion of glucose might be advanced as an explanation of the abnormally high temperature. We have never been able to give glucose at the rate of 15 gm. per kilogram per hour and maintain the water balance, because the heart is not capable of propelling the necessary volume of fluid. Consequently, we have not been able to determine the rate at which glucose actually burns under these conditions. But we have given 10 gm. per kilogram per hour for a period of seven hours, maintaining the water balance, without the advent of a rise of temperature. (The injection of glucose at rates above 2 gm. per kilogram per hour, will be discussed in a paper on "Intravenous Injections of Glucose at Higher Rates," now being prepared.)

We also performed two experiments, dehydrating with hypertonic sodium chlorid-sodium carbonate solutions, to ascertain how high a temperature might be obtained by employing crystalloids which do not burn in the body.

Experiment 3.—A female bull-terrier, weighing 10.4 kilograms, was given intravenously 753 c.c. of a 1.4 per cent. sodium chlorid—1 per cent. sodium carbonate solution, over a period of three hours. During this period the dog passed only 255 c.c. of urine. There was, therefore, no absolute dehydration. The temperature, which was 102.2 F. per vagina at the beginning, did not change. The concentration of sodium chlorid was therefore raised to 5 per cent. During the next two hours and thirty-five minutes the dog received 800 c.c. of this new solution and passed 1,875 c.c. of urine. During the course of the five hours and thirty-five minutes of the total injection, the dog received 1,553 c.c. of fluid and passed 2,130 c.c. of urine, a net output over intake of 577 c.c. or 55.5 c.c. per kilogram. Convulsions began and the injection was stopped thirty minutes before death. The temperature rose from 102.2 F. to 113 F. A rise of 9 degrees occurred after the cessation of urinary flow.

Excessive muscular activity during the convulsions might be suggested as a cause of the high temperature. To eliminate this factor a similar experiment was planned and the animal fully narcotized with ether at the first signs of chill, thus preventing the advent of convulsions.

Experiment 4.—A dog, weighing 10.4 kilograms, received, for a period of three hours, 718 c.c. of a 5 per cent. sodium chlorid—1 per cent sodium carbonate solution, and passed 1,005 c.c. of urine. The fluid output exceeded the intake by 287 c.c. or 28.2 c.c. per kilogram. The first slight twitchings due to a beginning chill began thirty-eight minutes before the close and were immediately stopped by the anesthetic. The temperature rose from 101.3 F. to 111.2 F.

Fever in Poikilothermic Dogs.—In order to determine whether the temperature changes during these experiments were in any way grossly influenced by a nervous mechanism, several dogs were rendered poikilothermic by sectioning their spinal cords between the sixth and seventh cervical segments. These dogs were also observed for manifestations of chills. Several experiments were made before the results were satisfactory. Following section of the cord, the body temperature adjusts itself to that of the environment and, at ordinary room temperature, it falls steadily and only becomes constant again at a deeply subnormal level. Under such conditions glucose injections may fail to start diuresis. If diuresis does occur, and it is possible to dehydrate in the usual way, the body temperature may rise materially and still the final temperature may remain markedly subnormal so that one could not then speak of having produced fever in the absolute sense. Again, if the attempt is made to keep the body temperature from falling too low by means of heating appliances, there is danger that fever may be pro-

duced accidentally by the external heat, and it requires elaborate equipment to maintain an operating room constantly at the desired temperature. The simplest method, and one that proved entirely practical, was simply to swathe the animal in cotton or blankets immediately after the operation. In this way the temperature may be kept constant for hours at a level only slightly below the normal.

Experiment 5.—The spinal cord was sectioned as described above. During the next four hours the dog cooled, spontaneously, to 83.3 F. It was then taken to a room heated approximately to this temperature, 84.4 F. During the next three hours, the dog, weighing 11.5 kilograms, received intravenously 582 c.c. of a 70 per cent. glucose solution, and passed 765 c.c. of urine. The fluid output exceeded the intake by 183 c.c. or 15.9 c.c. per kilogram. The temperature rose from 83.3 F. to 93.2 F., a rise of 9.9 degrees. Slight twitchings of the muscles of the neck and face began just before the death of the animal. Otherwise the usual outward manifestations of chill were missing.

Experiment 6.—An attempt at dehydration, in an animal whose temperature had been allowed to fall to 77 F., failed on account of the lack of diuresis. An increase in temperature of only 1.8 degree was noted.

Experrment 7.—In this experiment the cord was severed as before. A covering was placed over the animal to prevent too great loss of heat. In the course of three and one-half hours from the time of the operation, the temperature gradually fell to 95 F., where it remained constant for four hours. During the next four hours the dog received intravenously 752 c.c. of a 61 per cent. glucose solution and passed 1,640 c.c. of urine. The fluid output exceeded the fluid intake by 888 c.c. or 71 c.c. per kilogram. The temperature rose from 95 F. to 106.7 F., an increase of 11.7 degrees. There were no external manifestations of chill and no convulsions.

These experiments prove that the fevers in question are not dependant on a nervous mechanism.

DISCUSSION

General Principles.—Because of its high specific heat, water is capable of absorbing large quantities of heat, thus preventing sudden high temperature rises in the cells.[8, 9, 10, 11] (It is a noteworthy fact that those tissues in which most heat is produced contain the highest percentage of body water.) Having absorbed heat from its sources of production, water, because of its fluid nature, is capable of distributing the heat equally throughout the body and carrying the excess to the surface where it may be given off. Water then acts as a buffer in high temperature changes, and as a vehicle of heat within the animal body.

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8. Mathews, A. P.: Textbook of Physiological Chemistry, New York, 1916.
9. Henderson, L.: The Fitness of the Environment, New York, 1910, p. 80.
10. Stewart, G. N.: Manual of Physiology, New York, 1914.
11. Hunt, E. H.: Regulation of Body Temperature in Extremes of Dry Heat. J. Hygiene **12**: 479, 1912.

Rubner,[12] Wolpert[13] and Zuntz[14] have established that, in a normal body, as heat production increases, heat elimination by radiation, conduction and evaporation also increase, but that the percentage of heat dissipation by evaporation continually increases as more heat is produced. In other words, normally the loss of heat through evaporation fully compensates for that which cannot take place through radiation and conduction in order to preserve the normal temperature. In fever, however, this is not true. Krehl and Matthes[15] found that during fever, although there was usually an increase of heat elimination by radiation and conduction as well as by evaporation, the loss by evaporation was not sufficient to maintain a temperature equilibrium as in a healthy body. This failure of evaporation to compensate for the loss of heat which cannot take place through radiation and conduction would indicate that something hinders the evaporation of water in fever. Either the total supply of water runs out or the water becomes more firmly bound in the tissues and less available for evaporation.

Further investigations show that when the compensatory loss of heat by evaporation is hindered, hyperthermia results. This has been demonstrated by Sutton[16] who subjected human beings to high temperatures in an atmosphere which contained enough moisture to prevent evaporation. Similar results were observed by Haldane[17] in miners working in warm, damp mines. That most fevers are caused by increased atmospheric temperature, and moisture is of course out of the question. This is true only in cases of sunstroke, heat prostration or the like.

How then may evaporation of body water be checked in other ways? One method of accomplishing this purpose lies in the actual removal of water from the body to the extent that not enough remains to carry off the excess heat. Theoretically, if all the water should be removed from the body the specific heat of all the tissues would be reduced, and with no means left of quickly conveying it to the surface heat would accumulate in the tissues of the body and a high pyrexia

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12. Rubner, M.: Die Beziehungen der Atmosphärischen Feuchtigkeit zur Wasserdampfabgabe. Arch. f. Hyg., 1890.

13. Wolpert, H.: Ueber den Einfluss der Lufttemperatur auf die im Zustand anstrengenden Körperlicher Arbeit ausgescheidenen mengen Kohlensaure und Wasserdampf beim Menschen. Arch. f. Hyg. **26**: 32, 1896.

14. Zuntz, N.: Ueber die Wärme Regulierung bei Muskelarbeit. Berl. klin. Wchnschr. **33**: 709 (Aug.), 1896.

15. Krehl, L. and Matthes, M.: Wie entsteht die Temperatursteigerung des fiebernden Organismus. Arch. exper. Path. u. Pharmakol. **38**: 284, 1896.

16. Sutton, H.: The Influence of High Temperature on the Human Body, Especially with regard to Heat Stroke. Path. and Bacteriol. **13**:62, 1908.

17. Haldane, J. S.: The Influence of High Air Temperature. J. Hygiene **5**: 494, 1905.

would result. This, of course, practically is an impossibility, for a certain amount of water must be present in order that chemical reactions may take place, but less extreme water deprivations, as confirmed by clinical and experimental evidence, inevitably lead to hyperthermias of various degrees.

The researches of Hunt[11] on the effects of prolonged perspiration indicate that there is a large reserve of water in the body tissues which is called out in emergency. Though a large amount of water may be lost without any change in temperature, this investigator believed as a result of his work, that exhaustion of the reserve water would have the same effect as prevention of evaporation by increased atmospheric moisture. So far as we are aware, he did not subject this view to direct experimental test. The present experiments do exactly this thing, and the results are in complete harmony with Hunt's conception.

Sugar Fever; Salt Fever.—This brings us to the discussion of sugar and salt fevers as described by the pediatricians. In 1899, Floyd Crandell[4] in an article on inanition fever credits McLane of New York with having first described the phenomenon of fever which develops in the new-born prior to the establishment of the regular flow of breast milk and subsides thereafter. McLane called it inanition fever, believing that the solids of the milk were what allayed it, but Crandell showed that water alone was equally effective, and that the condition was not due to inanition, but to thirst. Similar observations were made by Erich Mueller[18] concerning a form of fever which Halberstadt saw in infants undergoing changes of diet. In 1906, Schaps[19] reported that subcutaneous injections of small quantities of isotonic sugar or salt solution caused fever in normal infants reaching a crest in 8 to 10 hours to disappear within 24 hours. He reported that repetitions of the dose gave weaker results.

We have not seen anything comparable to what Schaps describes and mention it merely in passing as a type of sugar or salt fever which, if it occurs at all, is something different from that which we are here discussing,[20] but in 1908-9 Finkelstein[3] described fever after feedings of 100 c.c. of 12.5 per cent. lactose solution to infants with intestinal disorders. Other sugars and also salts produced the same results. Finkelstein's solutions were strongly hypertonic, his doses were large and his results have been amply confirmed. He made the

18. Mueller, E.: Durstfieber bei Saüglingen. Berl. klin. Wchnschr. **47**: 673, 1910.

19. Schaps, L.: Salz und Zucker Injektion beim Saügling. Gesellsch. f. Kinderh. **23**: 153, 1906.

20. The febrile reactions observed by Schaps and others with isotonic salt solutions have since come to be considered as due to the use of water which was not freshly distilled.

observation most significant in the present discussion, that these fevers could be made to disappear entirely by the administration of tea and even water alone. Thus, Finkelstein saw another kind of fever in infants which, like those described by McLane and Halberstadt, yield under water administration. However, Finkelstein was not clear as to the mechanism of his sugar and salt fevers, and ventured the opinion that they arose from "injuries to cells" caused by the physico-chemical effects of the sugar or salt. Finkelstein thought the cells were injured by physicochemical effects of the sugar or salt rather than by chemical effects, and that much is important. Following Finkelstein a number of communications on the same subject appeared which confirmed the facts but confused more than they clarified the question of mechanism, except the writings of Heim and John,[5] and of Peteri.[6] In summing up the literature in the light of his own observation, Peteri, in 1914, concluded that infection played no part, that external conditions of the atmosphere had been excluded, that the immediate causative factor is desiccation and that the height of the fever attained is in inverse ratio to the body weight. All of these conclusions are in perfect accord with our own reading of the literature, and the results of the present experiments bring proof of their correctness.

Heim and John concurred with Finkelstein's observation that sugars and salts operate to produce fever by their physicochemical effects, which is obvious, for when fever is produced by intravenous injections of lactose, every gram of the lactose given is recoverable unchanged in the urine. The lactose molecule has not been altered. It has done its work as a molecule. But Heim and John went further. Instead of saying that the sugar or salt acted in some unknown physicochemical way on the cells causing the cells in some other unknown way to produce fever, thus begging the question, they suggested that the molecule (of salt) enters the cell and increases the affinity of the cell for water and that it does it in this way. Salt molecules have an affinity for water, and if several hundred of them migrate into a cell, the cell containing them becomes endowed with an increased affinity for water just because it has more salt in it.

This is the conception which Hofmeister first expressed in explanation of the power of a salt in suitable concentration to increase the swelling of colloid jellies. Thus salt in the body holds water in the body by its "hydropigenous" (or edema producing) tendency. As a result, the elimination of water through the lungs and skin falls, cooling is retarded and the body becomes overheated by the continuance of its own metabolic fires. And, it may be added, that as the body warms up more and more, the metabolism becomes faster and faster; a vicious circle is established.

It is unnecessary in this place to delve further into the nature of the affinity of molecules of salt or sugar for water, or to discuss theories of the character of the physico chemical unions of salts or sugars and water in general. It is enough that salts and sugars generally do tend to hold water in association with themselves, and that these substances when added to a beaker of water increase its boiling point and retard its evaporation at any given temperature and pressure. The same may occur in the body. As previously pointed out, the work of the pediatricians with salt fever has been conducted on infants which were in all probability considerably dehydrated to begin with, for as Peteri noted, the heights of the fevers resulting from sugar or salt administrations appear to be related in inverse ratio to the body weight, while fluctuations of body weight are determinated largely by the water balance. Moreover, water stops the fever. Our own experiments demonstrate sharply that single large injections of sugar do not, as a rule, cause fever unless the animal is first depleted in water. Therefore, it seems clear that Hunt is right — that there is normally a reserve of water in the body; that a certain fraction of this may become bound by sugars or salts added to the body, but that this binding of water will not ordinarily exhaust the whole free water reserve and determine fever unless the water reserve has been depleted beforehand by actual removal of water from the body as by continued elimination through the lungs, skin, urine or bowels without replacement.

We believe that the discussion up to this point shows that "inanition" fever of infants, the fever seen by Halberstadt in infants during alterations of diet, sugar and salt fever in infants, in adults and in animals, are all thirst fevers due to a retarded evaporation of water caused by exhaustion of the body reserve of water available for evaporation at the normal body temperature. One cannot help asking the question: How many more of the fevers which we know in the clinic can also be drawn into the same category?

A PHYSICOCHEMICAL THEORY OF FEVER

We have entertained the following proposition:

Fever, *the symptom* as seen in typhoid, malaria, pneumonia, tuberculosis, rheumatism, measles, serum reactions, proteose intoxication and all ordinary febrile diseases, except insolation and the like, may mean a deficit of "free" water in the body. By the term "free" water we would convey the idea of water in states comparable to those of liquid water at from 20 to 40 C. and ordinary pressures. As A. P. Mathews points out in his book on Physiological Chemistry, 1915, pp. 190-191, the works of Eötvös, Ramsay and Shields and Armstrong, show that

in liquid water at from 20 to 40 C. there probably exist several kinds of molecules ranging in formula from $(H_2O)_2$ to $(H_2O)_4$. But in any case, in the present connection "free" water is used to suggest liquid water, capable of absorbing excess heat from the cells, conveying it via the blood to the surfaces of the body and there dissipating it by evaporation, all at the normal body temperature.

Over against "free" water the body contains "bound" water. By this term is meant water molecules associated in the form of hydrates with molecules of other substances such as salts, sugar, protein, etc.; also, of course, water in true chemical combination, but especially the hydration water in colloids. This would correspond to the water reserve of Hunt. It is assumed that the "free" water tends to be in equilibrium with the "bound" water, and that there is a tendency toward the maintenance of a certain "free" water concentration in the blood more or less analogous to the hydrogen ion concentration of the blood, or the blood sugar concentration. When a certain quantity of "free" water is eliminated from the body, its place is filled by more water liberated from the hydrate reserves, and when an excess of free water is taken into the body it is stored as hydrate water or eliminated from the body.

As the colloids are extremely sensitive and prone to change in their capacity to hold water in response to the subtlest chemical and physicochemical influences, it is easy to conceive how the maintenance of a uniform "free" water concentration in the blood might be susceptible to a certain degree of vasomotor nervous control. Thus, the vasomotor nerves, by constricting blood vessels in the liver and causing a relative asphyxia in the liver may increase the production of acid in that organ and so liberate more free glucose from its storage form — glycogen. In an analogous way vasomotor nerves might cause colloids to take up or release more water by causing changes of cell metabolism through variations in the blood supply as needs arose. But the same thing could be accomplished directly by physicochemical and chemical agencies without the assistance of nerves, and nerves could not regulate temperature unless there were free water and responsive cells for the nerves to influence.[21]

It is proposed that in the ordinary febrile diseases, such as typhoid, tuberculosis and others, the symptom fever is due to a deficit of "free" water resulting from an abnormal tendency on the part of the colloids of the body to bind water. The poison of the disease leads to changes of the cell colloids and increases their hydration capacities, so that they

21. The reader will note that we are discussing fever and the means of the body to prevent hyperthermia or excess of heat; we are not discussing other phases of the problem of regulation of the body temperature.

tend to take up and bind more water. The effect of this on the "free" water of the body is thus the same as that of thirst or the introduction of salt or sugar into the body from without.

It would follow from the above that if we measured the "free" water of the blood in fever, it would be found low even if the total water content of the body were high. It is known that the blood as a whole does commonly become concentrated in fever, but as yet we have no ready method of measuring the "free" water fraction, although means by which this might be accomplished suggest themselves. It would also follow that the cells should be found swollen, the secretions concentrated, etc., which, of course, is the general rule in fever (cloudy swelling of the parenchymatous organs). The thirst during fever and the sudden release of water in the form of urine and perspiration when fever ends by crisis point directly to an abnormal binding of water during the period of elevated temperature. Everyone is familiar with the remarkable emptying out of water via the kidneys and skin which may follow the crisis in a case of pneumonia. Liters of water may thus be liberated in a few hours, giving visible proof of the water retention of the febrile stage. Finally it would follow that if enough water were introduced into the body during fever to saturate the increased affinities of the colloids and provide an excess of free water, then fever should disappear entirely. Now it is possible by copious water administration to lower high grade fevers and to make low grade fevers disappear. The value of plenty of water in fever, and especially in the management of tuberculosis, is too well recognized to require discussion, but probably no one has yet daringly pushed the administration of water in fever to the ultimate limits which might be necessary to ascertain whether every fever can be made to disappear entirely if enough water is given.

The question as to whether it would be good for every patient who presents the symptom fever to eliminate this symptom by means of copious water administration if that were possible, is a question that has been raised repeatedly by friends to whom the present views have been presented. We may be pardoned, therefore, if we emphasize the fact that this question is not under discussion in the present paper. The first objective is to determine whether the symptom fever can or cannot be controlled in this way in a large percentage of cases, and the wisdom of doing so if possible would then remain to be determined by sufficiently numerous experiments in various special types of cases. It is quite conceivable that in some cases the tissues might be so badly poisoned that they would partially liquefy if sufficient water were supplied, and still leave no "free" water available for evaporation at 37 C. The administration of water might then be pushed until mas-

sive edemas or general anasarca supervened before a reserve of free water could be established, or it might prove impracticable to accomplish the desired result at all. These are questions to be answered by experiments.

Up to the present time we have conducted three experiments in pneumonia patients in all of which the body temperature returned to normal within twenty-four hours with the administration of eight liters or less of water. Several observations on diphtheria cases were indecisive, fever persisting in one case after the administration of ten liters of water. A dog poisoned with diphtheria toxin, on the other hand, ran a persistent temperature between 104 and 105 F., until given a large intravenous injection of 3 per cent. glucose solution, during which the temperature fell to normal within five hours. Other work is in progress. So far the results are considered as indecisive.

In closing, attention is invited to the recent work of Lillian Moore,[22] who repeats the experiments which have formed the entire basis for the conception that there are nervous "heat centers" in the brain which, when stimulated, may cause fever.[23] Her results were negative and she draws attention to the absence of any sound experimental support for the current teaching that ordinary fevers are due to derangements of any demonstrable nervous mechanism. She points to the need for a physicochemical theory of fever. The theory outlined above may, therefore, serve to fill a gap. It has at least the advantage that all of the factors which enter into it are concrete things that may be subjected to measurements, and it should be capable of sharp proof or disproof by well designed experiments without recourse to opinion. Whatever the outcome of such experiments, they will, perhaps, add something to our knowledge of the problem.

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22. Moore, Lillian: Normal Temperature Variations and the Temperature Effects of Operative Procedures. Am. J. Physiol. **60**:24, 1918. Relation of the Corpus Striatum to the Regulation of Body Temperature. Am. J. Physiol. **46**: 253, 1918.

23. The Regulation of body Temperature. Ed. J. A. M. A. **71**:1139 (Oct.), 1918.

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CLOTHING OF INFANTS IN SUMMER AND WINTER

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McClure and Sauer[1] emphasized the fact that in the study of the relation of summer heat to infant mortality the heat regulatory power of the infant has not received its due consideration. It is evident that a number of factors are at work to maintain a proper heat balance; on the one side such as influence the heat production, on the other such as determine its elimination. We are indebted to Rubner[2] and his co-workers for the most important data about the influence of clothing on heat loss. At moderate room temperature a certain amount of clothing reduced the heat loss by conduction and radiation to one-third of the loss through the bare skin. With a temperature of 37 C. the interference with the heat loss may be so great as to cause serious disturbances. This is in the adult who has at his disposal the potent heat regulation of visible perspiration. The infant, with its relatively greater body surface, is usually devoid of most of this regulatory mechanism up to a certain age. Excluding a few minor means of heat dissipation, the heat loss is dependent here on the evaporation of water from the lungs, the so-called insensible perspiration, and conduction and radiation. It is an open question to what degree the evaporation of water from the lungs of the infant can participate in the elimination of heat. The results of McClure and Sauer indicate that the insensible perspiration is of great importance, particularly with high room temperature. Conduction and radiation cannot function without a difference between the surface temperature and that of the surrounding air. With usual hospital clothing the heat loss from the covered areas by these means is very markedly reduced at a room temperature of 31 C.[1] In this connection an influence of clothing on the heat loss is evident. The relative importance of the various factors entering in consideration under various conditions has not been determined with any degree of accuracy. Nevertheless, it is clearly of importance to gain as much information as possible about any one factor exercising an influence on any of the mechanisms of heat loss. Very few data are available with reference to clothing, although

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1. McClure and Sauer: Am. J. Dis. Child. **9**:490, 1915; Am. J. Dis. Child. **10**:425, 1915; Arch. Int. Med. **21**:428, 1918.

2. Rubner: Lehrb. d. Hygiene, 1907, Ed. 8.

it is recognized that the interference of clothing with heat loss may be a factor in the morbidity of the infant from gastro-intestinal disorders.

I determined the weight of the clothing of 400 infants from 1 week to 2 years of age. The infants were brought to the infant welfare stations, and they represent the tenement population. It was their first visit to the station. In 200 cases, hot days of the summers of 1914, 1915 and 1916 were chosen; and in 200, cold days of the winters of 1914, 1915 and 116. None of the mothers had received any instructions about clothing their infants. Only the clothing worn regularly indoors was weighed. The fabric of which the clothes were made was almost without exception cotton. It is impossible to discuss the importance of various kinds of clothes and fabrics in the case of

TABLE 1.—Results of Weighing Infants in Summer and in Winter

Group	Series	Name	1 Age in Mos.	2 Feeding	3 Body Weight in Gm.	4 Indoor Clothing Weight in Gm.	5 Per Cent. of Clothing to Body Weight	6 Outdoor Temperature, F.	Remarks
I	Summer	G.	1	Breast	3,636	460	12.7	92	Ileocolitis, toxic
		E.	2½	Breast	4,772	530	11.1	92	Diarrhea
		K.	2⅔	Bottle	4,318	500	11.3	92	Blood in stools
	Winter	Z.	1	Bottle	4,160	200	4.8	26	Well
		F.	1½	Bottle	3,380	230	6.8	26	Well
		B.	1½	Bottle	2,590	400	15.0	26	Well
II	Summer	J.	6	Breast	7,010	270	3.8	85	Well
		G.	4	Bottle	5,530	1,090	19.0	85	Well
		H.	7	Bottle	7,550	289	4.0	85	Well
	Winter	S.	7	Bottle	6,480	1,000	15.4	28	Well
		L.	5	Bottle	7,750	700	9.0	28	Well
		M.	8	Breast	7,100	460	6.5	28	Slight cold
III	Summer	G.	14	Bottle	8,863	400	4.5	85	Well
		C.	11	Bottle	8,800	120	1.3	85	Well
		G.	15	Bottle	9,000	400	4.4	85	Well
	Winter	Q.	18	Bottle	11,150	400	3.7	22	Well
		C.	10	Breast	8,050	300	3.7	22	Cough
		L.	12	Bottle	9,450	320	3.4	22	Well

infants because no accurate data are available. The material used for the clothing of our infants and the way it was put on was rather uniform. For these reasons the weighing of the clothing is the most convenient method of obtaining comparable data. The question whether there are marked differences in the amount of indoor clothing worn by babies in summer and winter is of interest in itself. The further purpose was to see whether a relationship could be established between excessive clothing and gastro-intestinal disorders. The latter phase of the subject had to be abandoned for the obvious reason that when it seemed necessary the mothers were immediately instructed to modify the clothing. Very few of the infants came to the stations

with sickness such as diarrhea, etc. The number of observations is much too small to be utilized for this latter purpose. The infants were not selected, and only in a few cases had extra clothing been put on the child on account of sickness. By far the greater majority of the infants were not hospital or dispensary cases.

The tabulation of each of the 400 cases would be rather superfluous. They were divided into three groups according to weight: Group I comprises infants weighing up to 5 kg.; Group II infants weighing from 5 to 8 kg. (both inclusive); and Group III infants weighing more than 8 kg. Table 1 gives a few examples of each group. In the fifth column the amount of clothing in grams is given per 100 gm. of body weight. In this way results can be compared with greater ease. It might perhaps be better to determine the clothing with reference to the body surface, at the same time taking in consideration the relation of the clothed surface to the surface left

TABLE 2.—Summary of Results in Maximum and Minimum Values

Group	1 Number of Infants	2 Average Body Weight in Kg.	3 Average Clothing Weight in Gm.	4 Per Cent. of Aver. Clothing Weight to Aver. Body Weight	5 Maximum Clothing Weight in Gm.	6 Minimum Clothing Weight in Gm.	7 Maximum per Cent.	8 Minimum per Cent.
I. Infants up to 5 kg.								
Summer	38	4.2	365	8.7	710	100	19.2	2.1
Winter	63	4.0	362	9.1	1,050	170	37.5	5.2
II. Infants from 5 to 8 kg.								
Summer	105	6.5	353	5.4	1,090	100	19.7	1.5
Winter	82	6.4	415	6.5	1,000	110	15.4	1.8
III. Infants more than 8 kg.								
Summer	57	9.2	371	4.0	560	120	5.2	1.3
Winter	55	9.5	463	4.9	1,500	280	15.2	2.6

uncovered. Columns 2, 3 and 4 of Table 2 contain the summary of the results; columns 5, 6, 7 and 8 the individual maximum and minimum values. The first group includes infants up to three or four months. In this group the difference between the summer and winter clothing is practically nil, and the proportion of the weight of clothing to body weight is higher than in Groups II and III. No doubt the custom of dressing infants in long clothes, regardless of any other consideration but age, is responsible for this result. The difference in the weight of winter and summer clothing in the second group, comprising infants from about three to ten months of age, is 62 gm. The summer clothing weighs about 14.9 per cent. (about one-seventh) less than the winter clothing. The third group comprises the older infants, whose

age rarely exceeded fifteen months. The summer clothing weighs 92 gm. less than the winter clothing, the difference being 19.9 per cent. (about one-fifth).

The difference between the body weights of the summer and winter infants has not been considered. In the first group the summer infants weigh about 5 per cent. more than the winter infants; in the second there is a difference of about 1.6 per cent. in favor of summer; and in the third the winter weights exceed the summer weights by about 3.2 per cent. In the absence of standards it is impossible to say whether the amount of clothing for winter or summer is approximately proper. The only figures available for comparison are those of Griffith.[3] The nurse in charge of the hospital dispensary conducted the weighings which were obtained during autumn and winter. The figures given by him served for the calculations as given below.

TABLE 4.—Results of Weighings in Dispensary

Age	Cases	Average Body Weight, Kg.	Average Weight of Clothing, Gm.	Clothing per Cent. of Body Weight
Up to 3 mos.	10	2.69	850	31.6
3 to 6 mos.	19	4.14	822	19.9
6 to 9 mos.	13	5.53	850	15.4
9 to 12 mos.	10	6.04	822	13.6
12 to 24 mos.	21	7.45	964	12.9

Griffith further determined the relation of this clothing to a mean gross weight of infants. This he obtained by the utilization of the statistics of various investigators. Calculated for the body weight (undressed) we get:

TABLE 4.—Relation of Clothing to Mean Gross Weight

Age	Griffith		Sauer	
	Weight of Infant	Clothing per Cent. of Body Weight	Weight of Infant	Clothing per Cent. of Body Weight
Up to 3 mos.	4.5	18.9	4.0	9.1
3 to 6 mos.	6.5	12.6	6.4	6.5
6 to 9 mos.	7.9	11.3		
9 to 12 mos.	9.1	9.0	9.5	4.9
12 to 24 mos.	11.0	8.8		

Under Griffith the figures obtained from his data are given, under Sauer the figures of my winter series are adjoined. The relationship of clothing to the body weight of the babies of Griffith is rather high. The reason for this is evidently the poor state of nutrition of

3. Griffith: Trans. Am. Med. Assn., Section Dis. Child., p. 79, 1917.

his material. However, when the weight of the clothing as determined by him is brought in relation with the body weight of normal infants, the amount of clothing is much higher than that of the corresponding group of our children. It is possible that his infants were overdressed because they were below par. As Griffith implies, the decision whether a baby is underdressed or overdressed is left to the experienced eye. This means that the decision depends on custom, leaving out of consideration the possible vagaries of the experienced eye itself.

The fact that so little work has been done and that our results differ so greatly seems to make it extremely desirable that some standards, based on experimentation, be established with reference to rational infants' clothing. This should include the various materials and fabrics.

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